

## **p27<sup>kip1</sup> acts as a downstream effector of and is coexpressed with the $\beta_{1C}$ integrin in prostatic adenocarcinoma**

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### Article

Integrins are a large family of transmembrane receptors that, in addition to mediating cell adhesion, modulate cell proliferation. The  $\beta_{1C}$  integrin is an alternatively spliced variant of the  $\beta_1$  subfamily that contains a unique 48–amino acid sequence in its cytoplasmic domain. We have shown previously that *in vitro*  $\beta_{1C}$  inhibits cell proliferation and that *in vivo*  $\beta_{1C}$  is expressed in nonproliferative, differentiated epithelium and is selectively downregulated in prostatic adenocarcinoma. Here we show, by immunohistochemistry and immunoblotting analysis, that  $\beta_{1C}$  is coexpressed in human prostate epithelial cells with the cell-cycle inhibitor p27<sup>kip1</sup>, the loss of which correlates with poor prognosis in prostate cancer. In the 37 specimens analyzed,  $\beta_{1C}$  and p27<sup>kip1</sup> are concurrently expressed in 93% of benign and 84%–91% of tumor prostate cells. Forced expression of  $\beta_{1C}$  *in vitro* is accompanied by an increase in p27<sup>kip1</sup> levels, by inhibition of cyclin A–dependent kinase activity, and by increased association of p27<sup>kip1</sup> with cyclin A.  $\beta_{1C}$  inhibitory effect on cell proliferation is completely prevented by p27<sup>kip1</sup> antisense, but not mismatch oligonucleotides.  $\beta_{1C}$  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  $\beta_{1C}$  as an upstream regulator of p27<sup>kip1</sup> expression and, therefore, a potential target [...]

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# p27<sup>kip1</sup> acts as a downstream effector of and is coexpressed with the $\beta_{1C}$ 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  $\beta_{1C}$  integrin is an alternatively spliced variant of the  $\beta_1$  subfamily that contains a unique 48-amino acid sequence in its cytoplasmic domain. We have shown previously that *in vitro*  $\beta_{1C}$  inhibits cell proliferation and that *in vivo*  $\beta_{1C}$  is expressed in nonproliferative, differentiated epithelium and is selectively downregulated in prostatic adenocarcinoma. Here we show, by immunohistochemistry and immunoblotting analysis, that  $\beta_{1C}$  is coexpressed in human prostate epithelial cells with the cell-cycle inhibitor p27<sup>kip1</sup>, the loss of which correlates with poor prognosis in prostate cancer. In the 37 specimens analyzed,  $\beta_{1C}$  and p27<sup>kip1</sup> are concurrently expressed in 93% of benign and 84%–91% of tumor prostate cells. Forced expression of  $\beta_{1C}$  *in vitro* is accompanied by an increase in p27<sup>kip1</sup> levels, by inhibition of cyclin A-dependent kinase activity, and by increased association of p27<sup>kip1</sup> with cyclin A.  $\beta_{1C}$  inhibitory effect on cell proliferation is completely prevented by p27<sup>kip1</sup> antisense, but not mismatch oligonucleotides.  $\beta_{1C}$  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  $\beta_{1C}$  as an upstream regulator of p27<sup>kip1</sup> expression and, therefore, a potential target for tumor suppression in prostate cancer.

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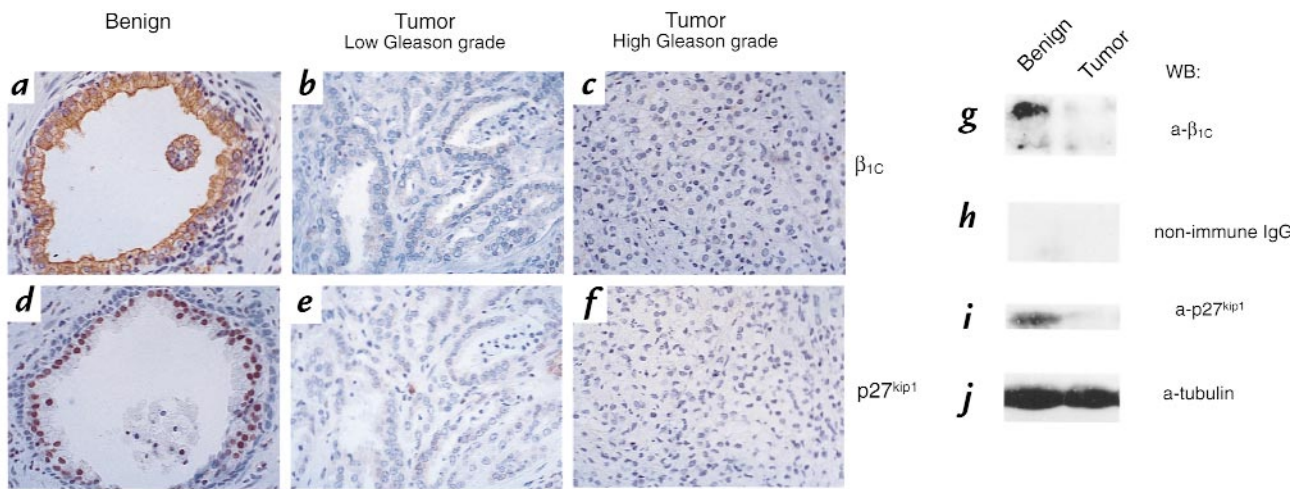
## Introduction

Integrins are a large family of transmembrane receptors composed of an  $\alpha$  and a  $\beta$  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  $\beta_{1C}$  integrin is an alternatively spliced variant of the  $\beta_1$  subfamily that contains a unique 48-amino acid sequence in its cytoplasmic domain (6). *In vivo*,  $\beta_{1C}$  is expressed in nonproliferative and differentiated epithelium (7). In the prostate,  $\beta_{1C}$  is found in benign glandular epithelial cells and is selectively downregulated in adenocarcinoma (8). Previous studies have shown that forced expression of either  $\beta_{1C}$  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<sup>kip1</sup>, 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<sup>kip1</sup> regulated expression is suggested by recent studies showing that in prostate cancer, as well as in breast or colorectal carcinomas, loss of p27<sup>kip1</sup> 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<sup>kip1</sup> and p21<sup>cip1/waf1</sup>, 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** Downregulation of  $\beta_{1C}$  and  $p27^{kip1}$  expression in prostatic adenocarcinoma. The expression of  $\beta_{1C}$  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  $\mu\text{g}/\text{ml}$  affinity-purified antibody to  $\beta_{1C}$  (a–c) or 0.6  $\mu\text{g}/\text{ml}$  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  $\mu\text{g}/\text{ml}$  affinity-purified antibody to  $\beta_{1C}$  (g), 2  $\mu\text{g}/\text{ml}$  nonimmune rabbit IgG as negative control for  $\beta_{1C}$  (h), or 0.8  $\mu\text{g}/\text{ml}$  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  $\beta_{1C}$  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*  $\beta_{1C}$  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  $\beta_{1C}$  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  $\beta_{1C}$  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  $\beta_{1C}$  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  $\beta_{1C}$  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  $\beta_{1C}$  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  $\beta$ -tubulin (Sigma Chemical Co., St. Louis, Missouri, USA); Ha2/5 to rat  $\beta_1$  integrin (PharMingen, San Diego, California, USA); TS2/16 to human  $\beta_1$  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  $\beta_{1C}$  and  $p27^{kip1}$  immunoreactivity. Serial sections from paraffin-embedded and formalin-fixed tissue specimens stained previously using antibodies to  $\beta_{1C}$  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  $\beta_{1C}$ , a

**Table 1**Correlation of  $\beta_{1C}$  and p27<sup>kip1</sup> expression in benign and neoplastic prostate

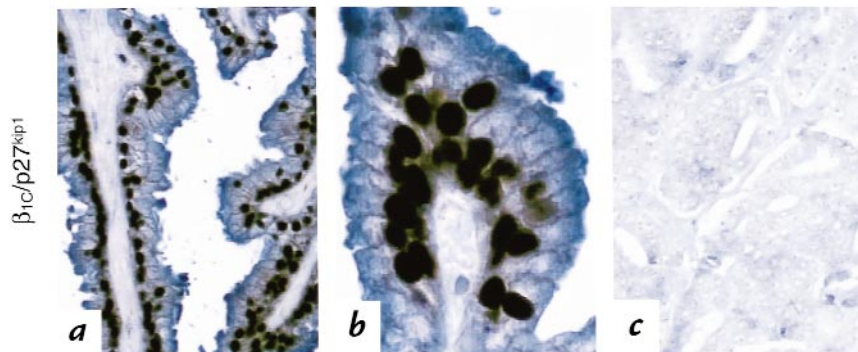
Benign				Tumor			
n*	%	$\beta_{1C}$	p27 <sup>kip1</sup>	n*	%	$\beta_{1C}$	p27 <sup>kip1</sup>
37	93 ± 5	+	+	7	84 ± 5.3	+	+
				24	91 ± 9.4	-	-
				1	0	+	-

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  $\beta_{1C}$  or p27<sup>kip1</sup> 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  $\beta_{1C}$  and p27<sup>kip1</sup> 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  $\beta_{1C}$  and p27<sup>kip1</sup>, as evaluated in double immunostaining experiments. The percentage of cells that showed correlation of  $\beta_{1C}$  and p27<sup>kip1</sup> expression was calculated as ratio of number of cells either expressing or lacking both molecules/total cell number counted in five fields  $\times 100$ .

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  $\beta_{1C}$  and p27<sup>kip1</sup> 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  $\beta_{1C}$  and p27<sup>kip1</sup> staining; a minimum of 300 cells per specimen were evaluated.  $\beta_{1C}$  and p27<sup>kip1</sup> 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  $\beta_{1C}$  and p27<sup>kip1</sup> expression was calculated as the ratio of number of cells either expressing or lacking both molecules/total cell number counted in five fields ( $\times 100$ ). There was 98% concordance among the observers' scores; in one instance, because of disagreement among the observers, the specimen

**Figure 2**

(a-c) Coexpression of  $\beta_{1C}$  and p27<sup>kip1</sup> in benign and neoplastic prostate tissue. Double staining for  $\beta_{1C}$  and p27<sup>kip1</sup> 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  $\beta_{1C}$  and p27<sup>kip1</sup>, respectively). Immunohistochemical analysis was performed as in Fig. 1. Hematoxylin counterstain was not used. The results show that  $\beta_{1C}$  and p27<sup>kip1</sup> expressions correlate in benign and neoplastic prostate.

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  $\mu$ g/ml insulin (Sigma Chemical Co.), 0.1  $\mu$ M 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  $\mu$ g/ml streptomycin, 100 U/ml penicillin (Gemini Bioproducts Inc.), and 0.1 mM nonessential amino acids (Life Technologies Inc.).

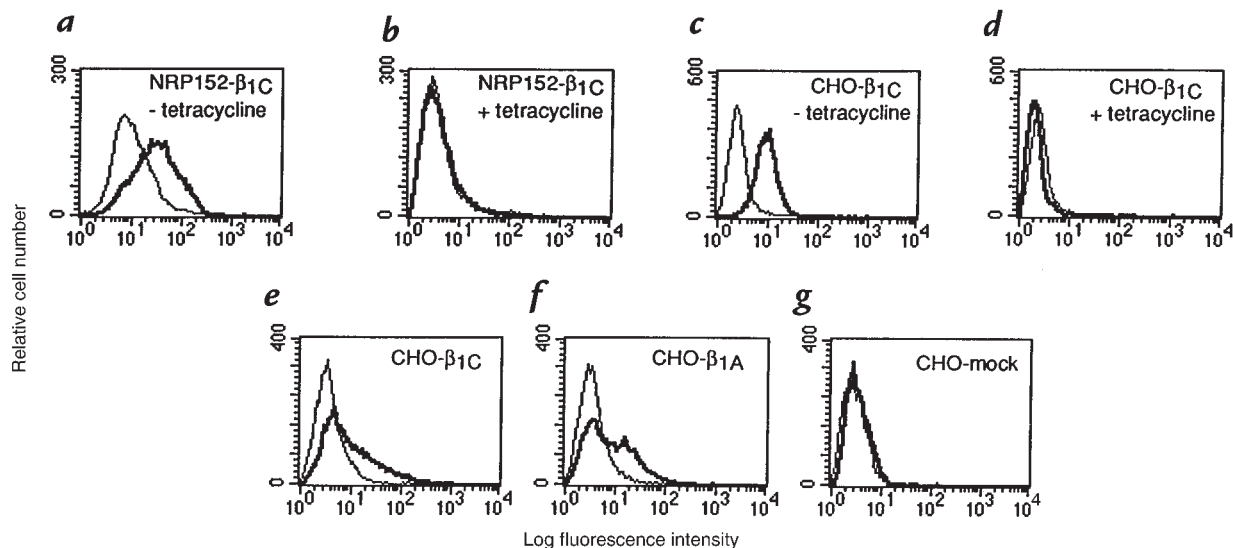
The tetracycline-regulated expression system, designed for the inducible expression of exogenous proteins in mammalian cells, consists of two plasmids: the pTet-tTA plasmid contains the neomycin-resistance gene and encodes a transcriptional transactivator (tTA) that drives expression of itself and a target gene inserted into the multiple cloning site of the second plasmid, pTet-Splice (27). To obtain stable transfectants expressing  $\beta_{1C}$  in a tetracycline-regulated system, *Clal-XbaI* fragment-encoding full-length human  $\beta_{1C}$  was isolated from Bluescript- $\beta_{1C}$  (9) and subcloned into *Clal-SpeI* sites in the pTet-Splice plasmid, a kind gift of D. Schatz (Yale University), to generate the pTet- $\beta_{1C}$  construct. NRP152 cells were electroporated using a GenePulser apparatus (Bio-Rad Laboratories Inc., Hercules, California, USA) set at 300 V and 950  $\mu$ F, using either 20  $\mu$ g pTet- $\beta_{1C}$  or pTet-splice, along with 10  $\mu$ g 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  $\beta_{1C}$  integrin by FACS<sup>®</sup>, using TS2/16, MAB against human  $\beta_1$  integrin, or 12CA5 MAB, as a negative control, as described (9). Stable transfectants were maintained in growth medium containing 1  $\mu$ g/ml tetracycline (Boehringer Mannheim) and 0.1 mg/ml G418.

The CHO cells were transfected as described above, either with the pTet- $\beta_{1C}$  construct or the pTet-splice, along with the pTet-tTA plasmid by electroporation, using a GenePulser apparatus set at 350 V and 950  $\mu$ F. 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  $\beta_{1C}$  integrin by FACS<sup>®</sup> analysis, and stable transfectants were maintained as described above.

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

CHO cell adhesion to fibronectin (3  $\mu$ g/ml), vitronectin (30  $\mu$ g/ml), BSA (10 mg/ml; Sigma Chemical Co.), and TS2/16 (1:10 dilution of culture supernatant) was performed as described previously (28) using [<sup>51</sup>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 3**

(a-g) Exogenous expression of  $\beta_{1C}$  in NRP152 and CHO cell transfectants. Stable cell transfectants expressing  $\beta_{1C}$  were generated using a tetracycline-regulated expression system. NRP152- $\beta_{1C}$  or CHO- $\beta_{1C}$  stable cell transfectants were cultured for 72 h, either in the absence (a and c) or in the presence (b and d) of 1  $\mu$ g/ml tetracycline and analyzed by FACS<sup>®</sup> using TS2/16, MAB to human  $\beta_1$  integrin, or 12CA5 as a negative control, followed by FITC-goat anti-mouse IgG. Fluorescence intensity is expressed in arbitrary units. FACS<sup>®</sup> analysis of a representative  $\beta_{1C}$  clone is shown. CHO cells were transiently transfected using pBJ1- $\beta_{1C}$  (e), or pBJ1- $\beta_{1A}$  (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  $\beta_1$  integrins, NRP152- $\beta_{1C}$  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<sup>kip1</sup> expression was evaluated by immunoblotting as described below.

To detect cyclin E, cyclin A, CDK2 or p27<sup>kip1</sup> 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  $\mu$ g/ml aprotinin (Sigma Chemical Co.), 10  $\mu$ g/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  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, and sonicated (30). Similar results were observed using either NP-40 or Tween-20 lysis buffers (29, 30). To analyze  $\beta_{1C}$  and p27<sup>kip1</sup> 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  $\mu$ g/ml leupeptin, 1 mM benzamide (Sigma Chemical Co.), 1  $\mu$ M D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) (Boehringer Mannheim), 10  $\mu$ g/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<sup>kip1</sup>, and tubulin was performed as described (31); immunoblotting of  $\beta_{1C}$  integrin was carried out as described (8).

Cyclin A or cyclin E were immunoprecipitated (31), and the

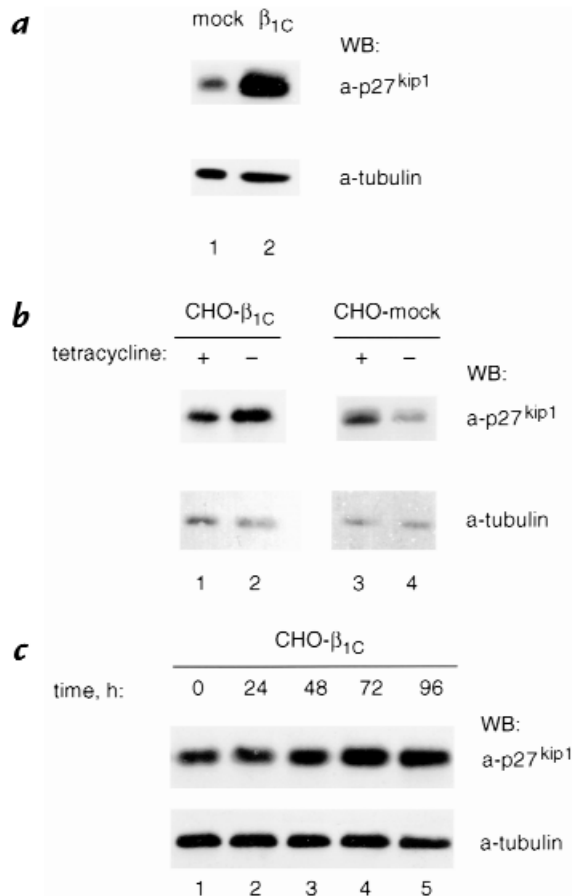
associated kinase activities were assayed as described (30), using 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol; Amersham Life Sciences Inc., Arlington Heights, Illinois, USA) and 33  $\mu$ g/ml histone H1 (Life Technologies Inc.) as a substrate. Phosphorylated histone H1 was observed using autoradiography.

The association of cyclin A with p27<sup>kip1</sup> was detected by immunoprecipitation with rabbit antiserum to cyclin A, followed by immunoblotting with MAB to p27<sup>kip1</sup>, 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  $\pm$  SEM. Group differences were compared using Student's *t* test.

**Oligonucleotide treatment and proliferation assay.** CHO- $\beta_{1C}$  cell transfectants ( $3.5 \times 10^3$ ) were plated on tissue culture plates in growth medium containing 1  $\mu$ g/ml tetracycline. After approximately 24 h, tetracycline was removed to induce  $\beta_{1C}$  expression. A mixture of 30 nM oligonucleotides (Gilead Sciences, Foster City, California, USA) (32) and 5  $\mu$ g/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  $\mu$ Ci [<sup>3</sup>H]thymidine per well (5.0 Ci/mmol; Amersham Life Sciences Inc.) during the last 3 h of the 48-h culture. [<sup>3</sup>H]thymidine incorporation was evaluated as described (9). In each experiment, duplicate or triplicate observations were performed, and the values are reported as mean  $\pm$  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<sup>kip1</sup> C-5-propyne-modified phosphorothioates used were 5'-UGGCUCUCCUGCGCC-3' and 5'-UCCCUU-UGGCGCGCC-3', respectively (32).



**Figure 4**  
 $\beta_{1C}$  expression is accompanied by increased p27<sup>kip1</sup> protein levels in NRP152 and CHO cells. (a) NRP152- $\beta_{1C}$  (lane 2) or NRP152- $\beta_{1C}$ -mock (lane 1) stable cell transfectants were cultured for 72 h in the absence of tetracycline, detached, and NRP152- $\beta_{1C}$  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<sup>kip1</sup> expression levels were evaluated by immunoblotting using 0.8  $\mu$ g/ml MAB to p27<sup>kip1</sup> (top). (b and c) CHO- $\beta_{1C}$  (b, lanes 1 and 2; c) or CHO- $\beta_{1C}$ -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  $\mu$ g/ml tetracycline. In these experiments cells were not detached and allowed to reattach to MAB to  $\beta_1$  integrins, but were lysed in the tissue culture plate, and p27<sup>kip1</sup> expression levels were evaluated as described in a. The experiments were repeated three times using two different  $\beta_{1C}$  clones with similar results. Group differences were compared using Student's *t* test. The differences in p27<sup>kip1</sup> expression levels in CHO- $\beta_{1C}$ , 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.

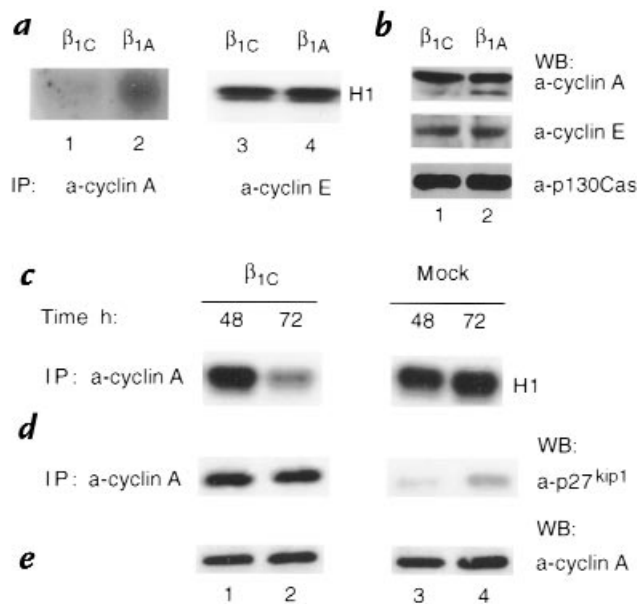
**MAP kinase mobility shift and immunocomplex kinase assay.** Serum-starved CHO cells, transiently transfected either with  $\beta_{1C}$  or  $\beta_{1A}$  integrins, were detached with 0.05% trypsin/0.53 mM EDTA. After trypsin neutralization by 1 mg/ml soybean trypsin inhibitor, cells were washed twice 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  $\beta_1$  integrin, either in the absence

or in the presence of 1% FCS, or with 10  $\mu$ g/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  $\beta_{1C}$  and p27<sup>kip1</sup> in prostatic adenocarcinoma.** Expression of  $\beta_{1C}$  and p27<sup>kip1</sup> was examined by immunohistochemistry and immunoblotting in 37 specimens resected for prostatic adenocarcinoma (Figs. 1 and 2; Table 1). Marked expression of both  $\beta_{1C}$  and, in the nuclei, of p27<sup>kip1</sup> 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  $\beta_{1C}$  and p27<sup>kip1</sup> 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. 2c and Table 1). Both  $\beta_{1C}$  and p27<sup>kip1</sup> 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  $\beta_{1C}$  and p27<sup>kip1</sup>, 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  $\beta_{1C}$  expression is accompanied by increased levels of p27<sup>kip1</sup>.** On the basis of these observations, we hypothesized that  $\beta_{1C}$  might have a causal role in regulating p27<sup>kip1</sup> levels *in vitro*. To test this hypothesis, we generated NRP152 or CHO transfectants expressing human  $\beta_{1C}$  under the control of a tetracycline-regulated promoter.  $\beta_{1C}$  expression in NRP152 (Fig. 3, a and b) or CHO (Fig. 3, c and d) cells was analyzed by fluorescence-activated cell sorter (FACS<sup>®</sup>) using either TS2/16, MAB to human  $\beta_1$  integrin (which does not cross-react with either rat or hamster  $\beta_1$  integrin), or 12CA5 as a negative control; in both cell types, maximal  $\beta_{1C}$  expression was obtained 72 hours after tetracycline removal (not shown). NRP152- $\beta_{1C}$  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  $\beta_1$  integrin. Immunoblotting analysis showed an increase of p27<sup>kip1</sup> expression in NRP152- $\beta_{1C}$  stable transfectants compared with mock-transfected cells (10.9  $\pm$  2.6-fold increase in two experiments; Fig. 4 a, top). Similar results were confirmed using CHO- $\beta_{1C}$  stable transfectants. In these experiments, cells were not detached and were allowed to reattach to monoclonal antibodies to  $\beta_1$  integrins, but they were lysed in the tissue culture plate. In these cells, we observed a 4.65  $\pm$  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  $\pm$  0.41-fold increase in three experiments; Fig. 4 b, compare lanes 3 and 4; top). The increase of p27<sup>kip1</sup> levels in response to  $\beta_{1C}$  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- $\beta_{1C}$  cell transfectants. (a) CHO cells were transiently transfected using pBJ1- $\beta_{1C}$  (lanes 1 and 3) or pBJ1- $\beta_{1A}$  (lanes 2 and 4). Total cell lysates, obtained as described in Methods, were immunoprecipitated using 1  $\mu$ g/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)  $\beta_{1C}$  expression does not affect cyclin E or cyclin A protein levels. Total cell lysates, obtained as described in a, were immunoblotted with 1  $\mu$ g/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- $\beta_{1C}$  (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  $\beta_{1C}$  clones with similar results. (d) Expression of  $\beta_{1C}$  is accompanied by increased p27<sup>kip1</sup> association with cyclin A. Total cell lysates were immunoprecipitated using rabbit antiserum to cyclin A, and the associated p27<sup>kip1</sup> 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  $\beta_{1C}$  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- $\beta_{1C}$  versus CHO- $\beta_{1A}$  transfectants are statistically significant (in a,  $P = 0.0001$ ; in c,  $P = 0.0069$ ).

hours (Fig. 4 c, lane 4). The results show that  $\beta_{1C}$  expression is accompanied by increased levels of p27<sup>kip1</sup> in both nontumorigenic as well as tumorigenic cells.

*Selective inhibition of cyclin A-dependent kinase activity and increased p27<sup>kip1</sup> association with cyclin A in  $\beta_{1C}$  transfectants.* Because p27<sup>kip1</sup> binds to and inhibits the activity of cyclin-CDK complexes (12), we investigated whether specific cyclin-associated kinase activities were inhibited in  $\beta_{1C}$  transfectants. Either transient transfectants or two independent stable clones expressing  $\beta_{1C}$  were used as controls for potential clonal variability. Cyclin A or cyclin E were immunoprecipitated from CHO cell lysates tran-

siently expressing  $\beta_{1C}$  or  $\beta_{1A}$  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 ( $53 \pm 3\%$  decrease in five experiments) in  $\beta_{1C}$  transfectants as compared with  $\beta_{1A}$  transfectants (Fig. 5a, compare lanes 1 and 2), whereas cyclin E-associated kinase activity was unaffected ( $1.5 \pm 0.98\%$  increase in two experiments; Fig. 5a, lanes 3 and 4). A  $70.5 \pm 1.5\%$  decrease in two experiments in cyclin A-associated kinase activity was also observed in stable transfectants upon maximal induction of  $\beta_{1C}$  expression at 72 hours (Fig. 5c, lane 2), whereas no effect was seen in mock-transfected cells (Fig. 5c, lane 4). A strong inhibition of cyclin A-associated kinase activity was also observed in NRP152- $\beta_{1C}$  transfectants (data not shown). Immunoblotting analysis of cyclin E, cyclin A, and CDK2 showed that  $\beta_{1C}$  expression had no effect on the levels of these proteins (Fig. 5b, top and middle, e; data not shown).

Because cyclin A-associated kinase activity can be inhibited by increased association of p27<sup>kip1</sup> to cyclin A-CDK complexes, we analyzed the amount of p27<sup>kip1</sup> associated with these complexes in  $\beta_{1C}$  transfectants. Comparable amounts of cyclin A were immunoprecipitated from total cell lysates of either  $\beta_{1C}$  or mock-stable transfectants cultured in the absence of tetracycline for 48 or 72 hours, and p27<sup>kip1</sup> association was analyzed by immunoblotting. A substantial increase in the amount of p27<sup>kip1</sup> associated with cyclin A was found in  $\beta_{1C}$  transfectants (Fig. 5d, lanes 1 and 2) versus mock-transfected cells (Fig. 5d, 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  $\beta_{1C}$  transfectants is not a consequence of a decrease in cyclin A expression, but is likely a reflection of its increased association with p27<sup>kip1</sup>. Expression of  $\beta_{1C}$  did not affect cell adhesion to ECM proteins, specifically  $\beta_1$  integrin ligands such as fibronectin and vitronectin, or to integrin-binding antibodies such as TS2/16 (data not shown). These data suggest that  $\beta_{1C}$  could exert its growth-inhibitory effect via an increase of p27<sup>kip1</sup> and reduction of cyclin A-associated kinase activity, without affecting cell adhesion.

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

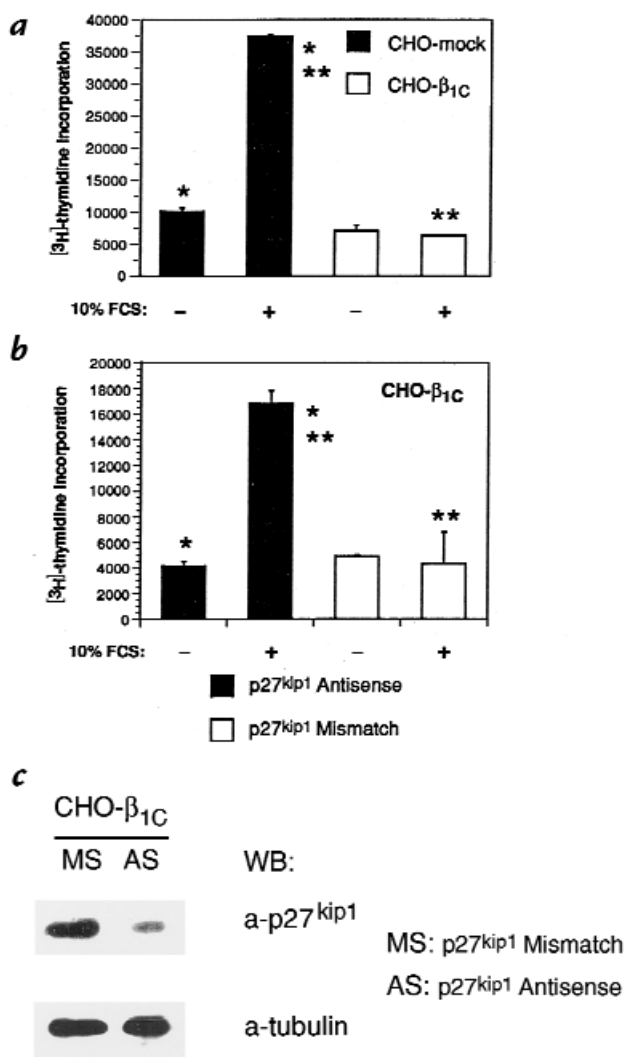
$\beta_{1C}$  stable transfectants in response to serum (Fig. 6b). These results show that p27<sup>kip1</sup> mediates  $\beta_{1C}$ -dependent growth inhibition.

**Forced  $\beta_{1C}$  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  $\beta_{1C}$  to modulate ERK-1 and/or ERK-2 activation, transient CHO cell transfectants expressing  $\beta_{1C}$  or  $\beta_{1A}$  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. 7a) and by *in vitro* kinase assays (Fig. 7b). A synergistic activation of ERK-1 and ERK-2 by  $\beta_{1C}$  or  $\beta_{1A}$  engagement and serum was observed when  $\beta_{1C}$  or  $\beta_{1A}$  transfectants were plated on TS2/16 in the presence of 1% FCS (Fig. 7a, lanes 4 and 6; b, lanes 2 and 4). Activation of ERK-1 and ERK-2 was not observed when  $\beta_{1C}$  or  $\beta_{1A}$  transfectants were plated on TS2/16 in absence of FCS (Fig. 7a, lanes 3 and 5; b, lanes 1 and 3), whereas  $\beta_{1C}$  transfectants plated on fibronectin were able to activate ERK-1 and ERK-2 (Fig. 7a, lane 7). Cells held in suspension either in the absence or in the presence (Fig. 7a, lanes 1 and 2, respectively) of 1% FCS showed very low levels of ERK-1 and ERK-2 activation. These data show that  $\beta_{1C}$  engagement does not prevent ERK-1 and ERK-2 activation in response to mitogenic stimuli or to fibronectin.

## Discussion

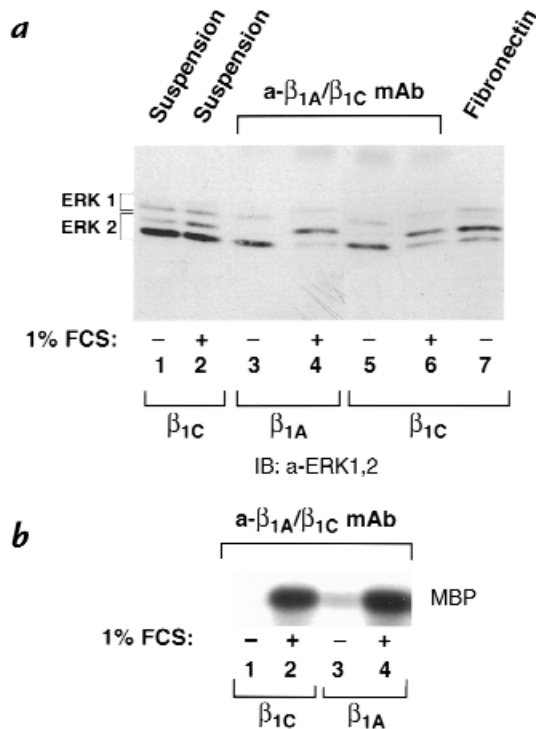
In this report it is shown, first, that *in vivo* expression of the  $\beta_{1C}$  integrin and of the CKI, p27<sup>kip1</sup>, correlates in benign and neoplastic prostate epithelial cells; second, that forced expression of  $\beta_{1C}$  *in vitro* is accompanied by increased levels of p27<sup>kip1</sup> and by selective inhibition of cyclin A-dependent, but not of cyclin E-dependent, kinase activity; third, that increased p27<sup>kip1</sup> association with cyclin A is observed as a consequence of  $\beta_{1C}$  expression; and fourth, that p27<sup>kip1</sup> mediates  $\beta_{1C}$ -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  $\beta_{1C}$  as an upstream regulator of p27<sup>kip1</sup>. Low levels of p27<sup>kip1</sup> 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<sup>kip1</sup> to evaluate response to therapy and differential treatment decisions has been recommended. Because *in vivo* downregulation of  $\beta_{1C}$  is likely to occur at an earlier stage than loss of p27<sup>kip1</sup> in the pathogenesis of prostate cancer we expect  $\beta_{1C}$  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  $\beta_{1A}$  integrin that is identical to  $\beta_{1C}$ , 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<sup>kip1</sup> antisense oligonucleotides prevent  $\beta_{1C}$  inhibitory effect on CHO cell proliferation. The experiments were repeated twice using two different  $\beta_{1C}$  clones with similar results. (a) CHO-mock or - $\beta_{1C}$  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  $\mu$ Ci/well [<sup>3</sup>H]thymidine was added during the last 3 h of the 48-h culture. (b) CHO- $\beta_{1C}$  stable cell transfectants were transfected using 30 nM of either p27<sup>kip1</sup> 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  $\mu$ Ci/well [<sup>3</sup>H]thymidine was added during the last 3 h of the 48-h culture. Results are mean  $\pm$  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- $\beta_{1C}$  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- $\beta_{1C}$  cells were transfected using 30 nM of either p27<sup>kip1</sup> antisense (AS) or mismatch (MS) oligonucleotides in the absence of tetracycline. After 24 h, the cells were lysed and p27<sup>kip1</sup> expression levels were evaluated by immunoblotting using 0.8  $\mu$ g/ml MAB to p27<sup>kip1</sup> (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  $\beta_{1C}$  engagement and serum stimulation. (a and b) Serum-starved CHO cells were transiently transfected using pBJ1- $\beta_{1C}$  or pBJ1- $\beta_{1A}$ . The cells were kept in suspension or seeded on tissue culture dishes coated with TS2/16 (a- $\beta_{1A}/\beta_{1C}$  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  $\mu$ g/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  $\mu$ g 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- $\beta_{1C}$  cells; lanes 3 and 4, CHO- $\beta_{1A}$  cells. In b, lanes 1 and 2, CHO- $\beta_{1C}$  cells; lanes 3 and 4, CHO- $\beta_{1A}$  cells. MAP, mitogen-activated protein; MBP, myelin basic protein; ERK, extracellular signal-regulated kinase.

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

Incorrect expression of integrins or of their cytoplasmic domain 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  $\beta_{1C}$  integrins in epithelial cells are found in benign and nonproliferative epithelium and are down-regulated in prostatic adenocarcinoma (7, 8). Forced expression of the cytoplasmic domain of  $\beta_{1C}$  has a causal role in inhibiting cell proliferation of tumorigenic and highly metastatic prostate cancer cells (7). Thus, because of the ability of  $\beta_{1C}$  to maintain high cellular levels of p27<sup>kip1</sup> it is expected that deregulation of  $\beta_{1C}$ , and consequently of p27<sup>kip1</sup> expression in prostate epithelial cells, may be an important step for malignant growth.

The effect of p27<sup>kip1</sup> antisense oligonucleotides shown in this study confirms the crucial role of p27<sup>kip1</sup> in modulating  $\beta_{1C}$ -dependent growth inhibition. The pathways controlled by  $\beta_{1C}$  specifically affect cyclin A-CDK activities, but not cyclin E-CDK activities, thus indicating that cyclin A-CDKs and p27<sup>kip1</sup> are selective downstream targets to this integrin. Although surprising, because of the p27<sup>kip1</sup> 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<sup>kip1</sup> preceded the observed decrease in cyclin A-associated kinase activity, thus pointing to p27<sup>kip1</sup> 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<sup>kip1</sup> in response to  $\beta_{1C}$  are being studied at this time. Unlike a previous report, where 10T1/2 fibroblasts expressing  $\beta_{1C}$  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<sup>cip1/waf1</sup>, which leads rectal carcinoma cells into an irreversible apoptotic pathway were observed in response to  $\beta_4$  integrin expression (38). Although we have not tested a potential effect of  $\beta_{1C}$  in stimulating apoptosis, this is unlikely to occur because  $\beta_{1C}$  expression appears to affect cell proliferation in a reversible manner. In fact, addition of tetracycline to the growth medium of  $\beta_{1C}$  transfectants did allow successful reculture of all cells, since cells expressing  $\beta_{1C}$  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<sup>kip1</sup> observed in  $\beta_{1C}$  transfectants was not accompanied by detectable changes in either cell adhesion to integrin ligands or spreading or focal contact organization; neither was dependent on  $\beta_{1C}$  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<sup>kip1</sup> 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<sup>kip1</sup> 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  $\beta_{1C}$  expression. Specifically, the failure of  $\beta_{1C}$  to prevent MAP kinase activation by fibronectin or by synergistic activities of  $\beta_{1C}$  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  $\beta_{1C}$  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,  $\beta_{1D}$ , an additional variant form of the  $\beta_1$  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  $\beta_{1C}$  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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