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Regulation of β_{1C} and β_{1A} Integrin Expression in Prostate Carcinoma Cells*

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β_{1C} and β_{1A} integrins are two splice variants of the human β_1 integrin subfamily that act as an inhibitor and a stimulator of cell proliferation, respectively. In neoplastic prostate epithelium, both these variants are down-regulated at the mRNA level, but only β_{1C} protein levels are reduced. We used an experimental model consisting of PNT1A, a normal immortalized prostate cell line, and LNCaP and PC-3, two prostate carcinoma cell lines, to investigate both the transcription/post-transcription and translation/post-translation processes of β_{1C} and β_{1A}. Transcriptional regulation played the key role for the reduction in β_{1C} and β_{1A} mRNA expression in cancer cells, as β_{1C} and β_{1A} mRNA half-lives were comparable in normal and cancer cells. β_{1C} translatio rate decreased in cancer cells in agreement wit decrease in mRNA levels, whereas β_{1A} transl increased more than 2-fold, despite the mRNA levels. Both β_{1C} and β_{1A} protei more rapidly in cancer than in no chase experiments showed th rates of β_{1C} and β_{1A} protei *versus* normal cells. In somal-mediated pr protein levels, the cells and the latter extent in cancer tha hibition of the ubiquit expression of ubiquitin ing β_{1A} protein levels in c that transcriptional, tra tional processes, the last inv lytic pathway, contribute to integrin, a very efficient inhi in prostate malignant transform

The integrin family of transmembrane receptors consists of heterodimeric glycoproteins composed of an α- and a β-subunit that, in addition to mediating cell adhesion to extracellular matrix proteins, play a pivotal role in regulating several cell functions, including proliferation, differentiation, migration, and intracellular signaling (1–5).

It is well established that the occurrence of alternatively spliced variants of both the α- and the β-subunit contributes to the variety of biological functions of the integrin receptors (6, 7). To date five different cytoplasmic variants have been identified for the human β_1 subunit, namely β_{1A}, β_{1B}, β_{1C}, $\beta_{1C\text{-}2}$, β_{1D}, and they have been sho to differentially affect receptor localization, cell prolifer hesion and migration, interactions with intracel as well as phosphorylation and activation (6, 8–13).

The β_{1C} i spliced variant of the β_1 integri que 48-amino acid se- his splice variant, at . the β_{1A} integrin, n prostate cancer fibroblasts (12, y expressed in 18) whereas 1A, the β_{1C} na (15, 18, , and inversely on in breast carci- mens, it was shown that n is down-regulated (15, 18, n levels are reduced or even lost y, we have shown that a reduction in activity of the β_1 integrin gene plays a role ism of down-regulation of β_{1C} and β_{1A} mRNA prostate adenocarcinoma tissue (22). Despite these minary findings, the molecular events that regulate the xpression of β_1 integrin variants in different pathophysiological conditions remain obscure.

Studies concerning the regulation of β_1 integrin expression have been previously reported in which probes and antibodies used did not discriminate among different cytoplasmic splice variants. In this regard, β_1 integrin expression was reported to be regulated at the level of transcription by cell attachment to the extracellular matrix (23), by transforming growth factor (TGF)-β1 treatment (24), and during differentiation (25) and cancer progression (26). On the other hand, a number of reports showed that expression of β_1 integrins is regulated both at the transcriptional and post-transcriptional/translational level (23, 27, 28). Moreover, post-translational mechanisms have been shown to play a role in regulating β_1 integrin expression levels: loss or reduction of β_1 integrins from the cell surface was associated with impairments in their glycosylation (*i.e.* maturation) process (25, 29, 30). Changes in protein stability have been involved in the regulation of integrin protein expression during carcinogenesis (31), but, to our knowledge, no report up to now has investigated the occurrence of changes in the rate of degradation of the β_1 integrins in different pathophysiological conditions.

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In light of the above findings, we investigated the regulation of the expression of β_{1C} and β_{1A} integrins at the transcriptional and post-transcriptional level, and as protein turnover and glycosylation in human prostate carcinoma cells. We show the following results. (i) A reduction in the transcriptional activity of the β_1 integrin gene can account for the down-regulation of β_{1C} and β_{1A} integrin mRNA levels in prostate cancer cells. (ii) Loss of β_{1C} protein in cancer cells depends not only on reduced transcription but also on reduced translation and increased protein degradation. (iii) Notwithstanding the reduction in the mRNA levels, the translation rate of the β_{1A} protein increases in cancer cells. (iv) The processing rates of β_{1C} and β_{1A} proteins are different in cancer *versus* normal cells. (v) The β_{1C} protein is a preferential target of the ubiquitin-proteasome proteolytic pathway in cancer cells.

EXPERIMENTAL PROCEDURES

Cell Culture—PNT1A cells, a human prostate normal cell line established by immortalization of normal adult prostate epithelial cells, LNCaP cells (clone FGC), a human prostate carcinoma cell line derived from a metastasis at the left supraclavicular lymph node, and PC-3 cells, a human prostate carcinoma cell line derived from a bone metastasis, were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). PNT1A cells were maintained in RPMI 1640 (Invitrogen Life Technologies, Milan, Italy) supplemented with 10% inactivated fetal bovine serum (FBS,[1] BioSpa, Milan, Italy), 2 mM glutamine (Invitrogen Life Technologies), 100 units/ml of penicillin (Invitrogen Life Technologies), and 100 μg/ml of streptomycin (Invitrogen Life Technologies), at 37 °C in the presence of 5% CO_2. PC-3 and LNCaP cells were maintained in RPMI 1640 supplemented with 1[illegible] FBS, 2 mM glutamine, 1 mM sodium pyruvate (Invitrogen Life T[illegible]ogies), 100 units/ml of penicillin and 100 μg/ml of streptom[illegible] in the presence of 5% CO_2.

RNA Extraction and RT-PCR—Cells were gr[illegible]ence, washed twice with cold PBS and total [illegible] using the TRIzol reagent (Invitrogen L[illegible] manufacturer's instructions. Whe[illegible] with the transcription inhibito[illegible] fore RNA extraction. Tota[illegible] sulting cDNA amplifi[illegible] System (Roche Applied [illegible] total RNA (1 μg) was reve[illegible] dNTPs (Roche Applied Scie[illegible] Milan, Italy), 1× RT-PCR bu[illegible] Science), 0.4 μM upstream prim[illegible] downstream primer (Invitroge[illegible] (AMV, TaqDNA polymerase an[illegible] Applied Science). After cDNA synth[illegible] for 2 min followed by 60 cycles o[illegible] annealing at 55 °C for 45 s and elong[illegible] The primer pair for β_{1A} was 2259-228[illegible]lining indicates the 5′-nucleotide position[illegible] size: 238 bp); the primer pair for β_{1B} was 2390-24[illegible]364 (predicted fragment size: 272 bp); the primer pair f[illegible]as 2441-2461 and 2592-2612 (predicted fragment size: 172 bp); t[illegible] primer pair for β_{1D} was 2442-2465 and 2562-2582 (predicted fragment size: 142 bp). All PCR reactions were performed using a Perkin Elmer CETUS PCR System (PerkinElmer Life Sciences). The PCR products were analyzed on a 2% agarose gel, stained with ethidium bromide.

Northern Blotting Analysis—Northern blotting analysis was carried out as described previously (22). Briefly, RNA samples (10 μg/lane) were separated on formaldehyde-agarose gels and blotted onto nylon membranes (Hybond N+; Amersham Biosciences). The blots were hybridized with cDNA probes, previously labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; PerkinElmer Life Science Products, Boston, MA) by random primer extension (Megaprime DNA labeling kit; Amersham Biosciences), for 20 h at 42 °C. The filters were washed once with 2× SSPE, 0.1% SDS for 10 min at room temperature, then with 1× SSPE, 0.1% SDS at 42 °C, followed by several washes in 0.1× SSPE, 0.1% SDS, at 65 °C and finally exposed at −80 °C overnight or longer to Kodak X-Omat AR 5 film (Kodak, Rochester, NY). Radiolabeled probes were generated using either the 116-bp fragment specific for β_{1C} integrin or the full-length human β_{1C} cDNA (19). Quantitative analysis was performed by densitometric scanning of the autoradiographs using a GS-700 Imaging densitometer (Bio-Rad); multiple exposures of the same Northern blots in a linear range were performed. 28 S rRNA signals were used as controls to determine the integrity of RNA and equality of loading in each lane. The average of either β_{1C} or β_1 mRNA expression levels in PNT1A cells was set at 100 (arbitrary units). β_{1C} and β_1 mRNA levels in prostate carcinoma cells were calculated as percentage of PNT1A mRNA levels, hybridized on the same filter. The mean value (±S.E.) of results obtained from at least three experiments was calculated.

Immunoblotting Analysis and Immunoprecipitation—PNT1A, LNCaP and PC-3 cells were grown to 60–70% confluence, washed twice with cold PBS and lysed in 150 mM NaCl, 20 mM Tris, pH 7.5, 1% Triton X-100, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μM calpain inhibitor. Where indicated, cells were incubated with either the protein synthesis inhibitor cycloheximide (12.5 μg/ml[illegible]) or the lysosomal inhibitors leupeptin (100 μM; Sigma) or [illegible]0 μM; Sigma) or NH_4Cl (50 mM), or the calpain inhibit[illegible] Calbiochem) or the ubiquitin-proteasome inhibi[illegible]iochem, La Jolla, CA) or the vehicle alon[illegible]tin, ALLN, MG132, Sigma; ethanol[illegible] protein content of each lys[illegible] protein assay reagent [illegible] extracts (100 μg) were [illegible]nder reducing condi-[illegible](PVDF) membranes [illegible]med as previously [illegible] affinity-purified [illegible]clonal antibody [illegible]g/ml of mono-[illegible]4 °C in Tris-[illegible]50 mM NaCl, [illegible]ee times in TBS-T [illegible]idase-conjugated goat [illegible]or mouse IgG (Amersham [illegible]emperature. After three washes [illegible] using the enhanced chemilumines-[illegible] Biosciences) according to the manufac-[illegible]sitometric values for immunoreactive bands [illegible] a GS-700 Imaging densitometer (Bio-Rad). β_{1C} [illegible]levels in prostate cancer cells were calculated as per-[illegible]e control (PNT1A cells), after normalization using β-tubu-[illegible]ntrol for protein loading.

[illegible]o perform a coupled immunoprecipitation-immunoblotting assay, the whole cell extract (1 mg of protein) was first precleared and then incubated overnight at 4 °C with either a 1:20 dilution of rabbit polyclonal antibody to β_{1C} integrin (18) or a 1:100 dilution of polyclonal antibody to β_{1A} integrin (Chemicon). Immunocomplexes were recovered by binding to protein A-Sepharose (Sigma) and washed three times with 350 mM NaCl, 20 mM Tris, pH 7.5, 1% Triton X-100, 1 mM PMSF, and twice with 150 mM NaCl, 20 mM Tris, pH 7.5, 1% Triton-X100, 1 mM PMSF. Immunocomplexes were analyzed by 10% SDS-PAGE under reducing conditions followed by transfer to PVDF membrane. Filters were immunoblotted using either a 1:1000 dilution of mAb to ubiquitin (Sigma) following the manufacturer's instruction, or 1 μg/ml mAb 13 to β_1 integrins (BD Biosciences-Transduction Laboratory, Temecula, CA), as previously described (19).

Nuclear Run-on Transcription Assay—Nuclear run-on assays were performed as described previously (22). Nuclei were isolated from 1×10^8 PNT1A, LNCaP, and PC-3 cells. *In vitro* run-on transcription was carried out by using 1×10^7 nuclei and 100 μCi of [α-^{32}P]UTP (3,000 Ci/mmole; PerkinElmer Life Sciences)/assay for 30 min at 30 °C, with periodic mixing. Labeled transcripts were purified by phenol/chloroform extractions and ethanol precipitations. A total of 1.2×10^7 cpm (4.0×10^6 cpm/ml of prehybridization solution) of elongated nascent RNAs per assay was hybridized for 48 h at 42 °C to filter-immobilized cDNAs. The following cDNA fragments were used: the 116-bp specific β_{1C} fragment (nucleotides 2435–2550) isolated by EcoRI digestion from the pBluescript-β_{1C} plasmid (19), the 2.6 kb full-length β_{1C} fragment, isolated by EcoRI digestion from the pBluescript-full-length β_{1C} plasmid (14), and a 1.3-kb 28 S fragment isolated by BamH1 digestion from the p28S

[1] The abbreviations used are: FBS, fetal bovine serum; ALLN, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal; AMV, avian myeloblastosis virus; cyx, cycloheximide; Me_2SO, dimethyl sulfoxide; dNTPs, deoxynucleotides; Endo-F, endoglycosidase F; mAb, monoclonal antibody; NRS, normal rabbit serum; PBS, phosphate-buffered saline solution; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; SSPE, saline sodium phosphate ethylenediaminetetraacetic acid buffer; CHO, chinese hamster ovary.

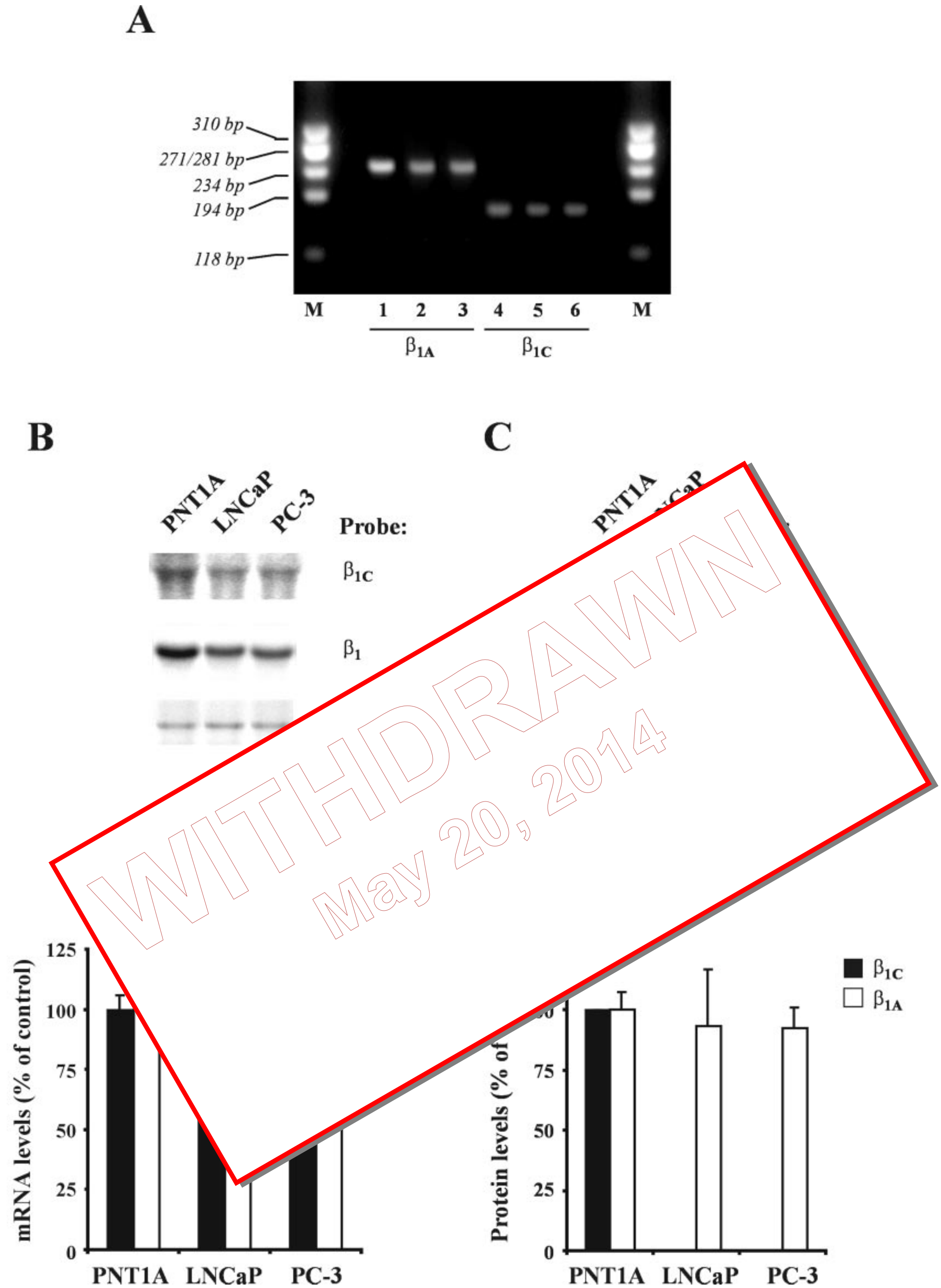


FIG. 1. **Expression of β_{1C} and β_{1A} integrins in carcinoma and normal prostate cells.** *A*, RT-PCR analysis of β_{1A} (*lanes 1–3*) and β_{1C} (*lanes 4–6*) mRNAs in PNT1A (*lanes 1* and *4*), LNCaP (*lanes 2* and *5*), and PC-3 (*lanes 3* and *6*) cells. In *lanes 1–3*, no amplification of β_{1C} in combination with β_{1A} was observed. *M*, molecular size marker. *B*, Northern blotting analysis for β_{1C} and β_1 integrin mRNAs. 28 S rRNA signals of the ethidium bromide-stained gel were used as controls to determine equality of loading in each lane. *Bottom*, β_{1C} and β_1 mRNA expression levels in LNCaP and PC-3 cells were calculated as percentage of PNT1A mRNA levels (*control*), set at 100. Mean values ± S.E. from three different experiments are shown. *C*, immunoblotting analysis of β_{1C} and β_{1A} integrin proteins. PNT1A, LNCaP and PC-3 cell protein extracts (100 μg) were electrophoresed on 10% SDS-polyacrylamide gel electrophoresis under reducing conditions, and immunostained using an antibody to either β_{1C} or β_{1A} integrin. β-tubulin signals were used as loading controls. *Bottom*, β_{1C} and β_{1A} protein expression levels in LNCaP and PC-3 cells were calculated as percentage of PNT1A protein levels (*control*), set at 100. Mean values ± S.E. from three different experiments are shown.

plasmid (32). After hybridization, the filters were washed once in 2× SSPE, 0.1% SDS for 10 min at 42 °C, twice in 1× SSPE, 0.1% SDS for 10 min at 42 °C, twice in 0.5× SSPE, 0.1% SDS for 10 min at 42 °C, once in 0.1× SSPE, 0.1% SDS for 10 min at 50 °C, and then exposed to Kodak X-OMAT AR 5 film (Kodak). Autoradiographs of the RNA-DNA hybrids were analyzed using a GS-700 Imaging densitometer (Bio-Rad). All values were normalized according to the signal of 28 S rRNA, used as an internal standard. The β_1 transcription rate in prostate carcinoma cells was calculated as percentage of the control (PNT1A cells).

^{35}*S-Metabolic Labeling*—The rate of β_1 integrin protein synthesis

and the stability of β_1 integrin proteins were determined in PNT1A, LNCaP and PC-3 cells maintained in culture on 100-cm dishes (~1×10^7 cells/dish). Cells were labeled for 3 h in 1 ml of methionine/cysteine-free RPMI 1640 medium (Sigma) containing 100 μCi/ml [^{35}S]protein labeling mix ([^{35}S]methionine/cysteine, Amersham Biosciences). Cells were washed twice with PBS and lysed with 100 μl/dish of lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.5, 1% Triton X-100, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μM calpain inhibitor) for 30 min at 4 °C. Cells were then scraped and insoluble material was removed by centrifugation at 10,000 × *g* for 30 min at 4 °C. In pulse-chase experiments, cells were incubated, before lysis, in fresh medium containing 10% FBS, supplemented with 10 mM methionine and 10 mM cysteine, for varying times (up to 96 h) after a 3-h pulse label. Cell lysates were precleared, and levels of trichloroacetic acid-precipitable radioactivity were determined (33). Trichloroacetic acid-precipitable counts per min (1×10^7) of precleared labeled cell extracts were immunoprecipitated overnight at 4 °C with either a 1:20 dilution of rabbit polyclonal antibody to β_{1C} integrin or a 1:100 dilution of polyclonal antibody to β_{1A} integrin or a 1:20 dilution of normal rabbit serum (NRS, Sigma), as negative control, and with either 50 μg/ml of mAb to β-tubulin or 50 μg/ml of non-immune mouse IgG (Sigma), as negative control. Immunocomplexes were recovered by binding to protein A-Sepharose and washed as described above. Immunocomplexes were analyzed by 10% SDS-PAGE under reducing conditions followed by either fluorography (Amersham Biosciences) and drying in a gel dryer (Bio-Rad) or transfer to PVDF membrane. Dried gels and PVDF membranes were exposed to Hyperfilm films (Amersham Biosciences) at −80 °C for 1–4 days. After autoradiographic exposure, PVDF filters were immunoblotted with 1 μg/ml mAb 13 to β_1 integrins as control of the total amount of β_1 integrins that were immunoprecipitated. Densitometric values for immunoreactive and radioactive bands were quantified using a GS-700 Imaging densitomet (Bio-Rad).

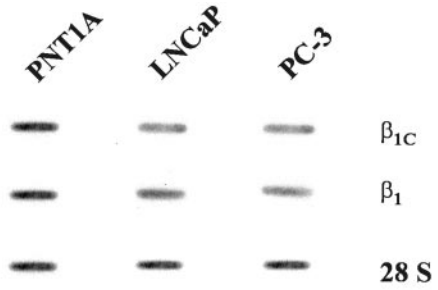
Endoglycosidase Digestion—Immunoprecipitates were di boiling for 4 min in 10 mM sodium phosphate (pH 7.0) SDS, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml l pepstatin, and 1 μM calpain inhibitor. Sample times with 100 mM sodium phosphate buff ase inhibitors, divided into two aliqu dase F (Endo-F; Roche Applied Sc Following a 12-h incubation was added, and samples incubations were term were subjected to 10% S sure to Hyperfilm films at

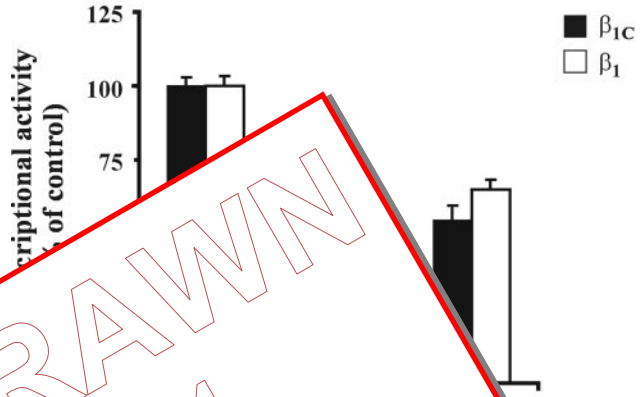
Statistical Analysis—Dat tical analysis was performed were repeated at least twice.

RE

β_{1C} and β_{1A} Integrin Expr Cells—To investigate the regula expression in prostate cancer cell (PNT1A), established by immortaliz tate epithelial cells, non-tumorigeni prostate cancer epithelial cell lines, L (clone FGC) (35, 36) and PC-3 (37, 38), were used as the experimental model.

First control, it was investigated by RT-PCR analysis whether β_{1C} and β_{1A} were the only β_1 integrin cytoplasmic variants expressed in prostate cells (Fig. 1*A*). This was confirmed by observing that only the β_{1A} sequence was detected when primers common to β_{1A} and β_{1C} were used, suggesting that β_{1C} mRNA is expressed at lower levels than β_{1A} mRNA in human prostate cells, as previously reported in prostate tissue (22) and in other cell types (14), and that an amplification product of the expected molecular size was detected both in normal and in carcinoma cells when primers specific for β_{1C} were used. By using specific primers to amplify the β_{1B} and β_{1D} transcripts, no amplification product was detected (data not shown), as in Ref. 22. In order to check whether the steady-state levels of β_{1C} and β_{1A} mRNAs and proteins were regulated in cancer *versus* normal cell lines in a way comparable to the prostate tissue, Northern (Fig. 1*B*) and Western (Fig. 1*C*) blotting analysis were performed in PNT1A, LNCaP, and PC-3





ns. Nuclear
1A, LNCaP,
-labeled nuclear
atured cDNA (10 μg)
had been immobilized.
NAs were normalized to the
bottom, the β_1 integrin transcrip-
3 cells was calculated as percentage of
PNT1A cells (*control*), set at 100, consid-
oradiographic signals. Mean ± S.E. from three
s are shown.

s shown in Fig. 1*B*, in LNCaP and PC-3 cells β_{1C} mRNA vels proved to decrease of 40.6% ± 3.3 ($p < 0.02$) and 55.7% ± 3.9 ($p < 0.02$), respectively, *versus* PNT1A cells. Decreases in β_1 mRNA, which reflects the β_{1A} mRNA, were also found for LNCaP and PC-3 *versus* PNT1A cells, 30.5% ± 2.9 ($p < 0.02$) and 35.5% ± 0.8 ($p < 0.02$), respectively. Fig. 1*C* shows that the β_{1A} protein was expressed in normal and carcinoma prostate cells at comparable levels, whereas, the β_{1C} protein was expressed in PNT1A cells, but was undetectable in either LNCaP or PC-3 cells.

These results show that β_{1C} and β_{1A} integrin mRNA and protein steady-state levels are regulated in LNCaP and PC-3 prostate cancer cell lines *versus* PNT1A normal cells in a way comparable to the prostate tissue.

Transcriptional Regulation of β_1 Integrins—In order to investigate whether, as previously reported in prostate tissue (22), a decreased transcription of the β_1 integrin gene could account for the down-regulation of β_1 integrin mRNA levels in prostate cancer cells, the β_1 integrin gene transcriptional activity was evaluated both in normal and carcinoma cell lines by nuclear run-on analysis, as described under "Experimental Procedures" (Fig. 2). The transcriptional rate of the β_1 integrin gene was found to be markedly reduced in LNCaP and PC-3 as compared with PNT1A cells: 40.3% ± 4.1 ($p < 0.01$) and 45.4% ± 4.7 ($p < 0.01$), respectively, when the β_{1C} autora-

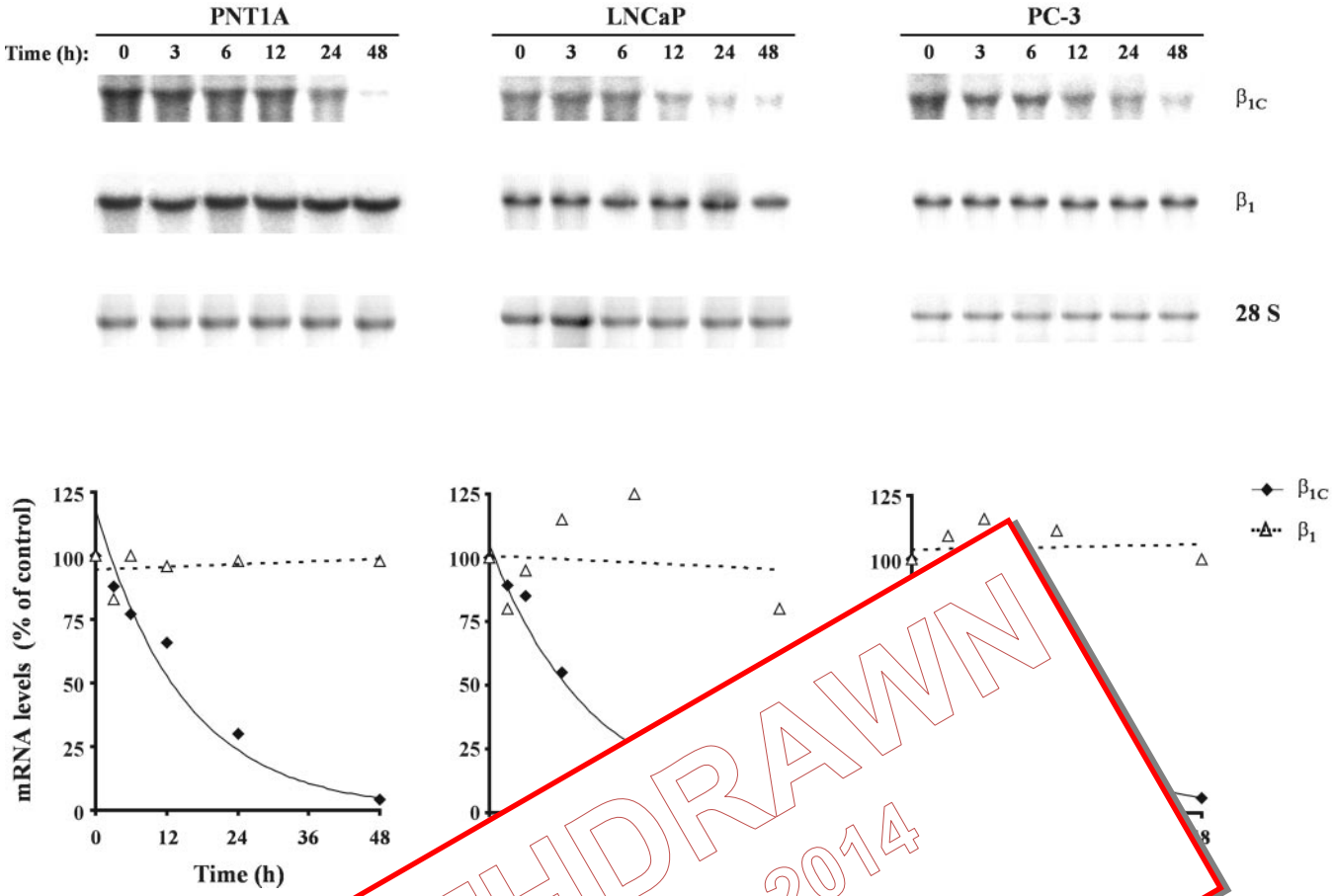


FIG. 3. **Post-transcriptional r** by addition of actinomycin D (7.5 μg/ml) to PN thern blotting up to 48 h after actinomycin D ad o normalize the amount of total RNA loaded for RNA expression levels were calculated as percentage in two independent experiments.

diographic signal was c integrin, an ^{35}S-labeled protein was and 35.1% ± 3.7 ($p < 0.$ with a similar molecular mass for the two was considered. variants (indicated by the *arrow*). A larger

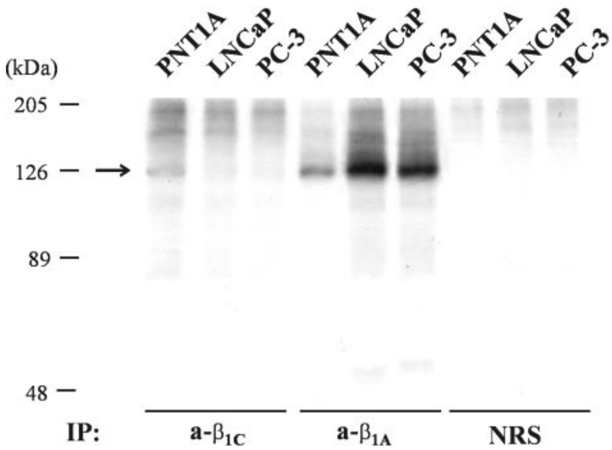
In order to ascertain wh β_{1A} than β_{1C} protein could be immunoprecipitated could contribute to the down- e PNT1A cell extracts, in agreement with the finding levels in prostate cancer cells, β at β_{1C} mRNA levels are lower than those of β_{1A} (Fig. 1*A*). In estimated by their steady-state le LNCaP and PC-3 cells, [^{35}S]methionine/cysteine incorporation into β_{1C} protein was reduced by 72.3% ± 5.4 ($p < 0.001$) and 74.9% ± 4.9 ($p < 0.001$), respectively, whereas [^{35}S]methionine/cysteine incorporation into β_{1A} protein was increased to 316.5% ± 44.7 ($p < 0.001$) and 251.7% ± 42.1 ($p < 0.001$), respectively, as compared with PNT1A cells. The translational rate of the housekeeping protein β-tubulin was also evaluated, as a control, and it was found comparable between normal and neoplastic prostate cells (data not shown). These results show that β_{1C} and β_{1A} integrin splice variants are translated with different efficiency in carcinoma cells.

actinomycin D, a transcription in the presence of actinomycin D, as meast de exclusion, was >97% in the three cell lin tinomycin D addition, a logarithmic decay was ob for β_{1C} mRNA levels, both in normal and neoplastic cells, but not for β_1 mRNA levels, that remained constant up to 48 h both in PNT1A and LNCaP and PC-3 cells (Fig. 3). No difference was detected in cancer *versus* normal cells with respect to β_{1C} mRNA half-life, which was ~12 h.

Overall, these results show that transcriptional but not post-transcriptional processes account for the down-regulation of β_{1C} and β_{1A} mRNA steady-state levels in prostate cancer cells.

Translational Regulation of β_{1C} and β_{1A} Integrins—Notwithstanding both β_{1C} and β_{1A} mRNA levels were down-regulated, only the β_{1C} protein was found to be reduced or lost in prostate cancer cells (Fig. 1 and Refs. 18 and 19). In order to establish whether a different modulation of the rate of β_{1C} and β_{1A} protein synthesis was involved in the regulation of β_{1C} and β_{1A} expression in prostate cancer cells, we assayed the translation process by metabolic labeling of PNT1A, LNCaP, and PC-3 cells with [^{35}S]methionine/cysteine, followed by immunoprecipitation studies (Fig. 4). Using a specific antibody to immunopre-

Stability and Glycosylation of β_{1C} and β_{1A} Proteins—In order to investigate whether changes in protein degradation rates could be involved in the differential regulation of β_{1C} and β_{1A} protein expression in prostate cancer cells, we measured the half-life of the two splice variant proteins. Firstly, translation was inhibited with cycloheximide and β_{1C} and β_{1A} steady-state protein levels were measured in normal and cancer prostate cells by immunoblotting analysis. Cell viability in the presence of cycloheximide was > 97% in the three cell lines, as measured by Trypan Blue exclusion. Fig. 5 shows that the β_{1C} protein levels declined logarithmically in normal cells, as well as the β_{1A} protein levels both in normal and neoplastic cells. The β_{1C}



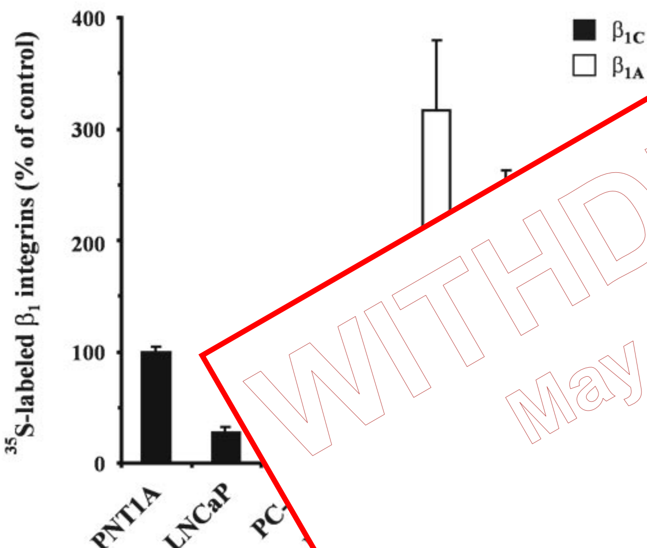


FIG. 4. **Translational regulati**[illegible] PNT1A, LNCaP, and PC-3 prostate [illegible] with [^{35}S]methionine/cysteine for 3 h, [illegible]pre-cipitated with an antibody to either β_{1C} [illegible] NRS, as negative control. Immunoprecipitated prot[illegible]phoresed on 10% SDS-polyacrylamide gel electrophoresis [illegible]ucing conditions. ^{35}S-labeled proteins were visualized by fluor[illegible]phy. Positions of molecular mass standards (kDa) are indicated. *Bottom*, the relative amounts of the nascent β_{1C} and β_{1A} integrins in LNCaP and PC-3 cells were expressed as percentage of β_{1C} and β_{1A} content in PNT1A cells (*control*), set at 100. Mean values ± S.E. from four independent experiments are shown.

half-life was ~12 h in PNT1A cells whereas the β_{1A} half-life was ~32 h in PNT1A and ~18 h both in LNCaP and PC-3 cells (Fig. 5*B*). Unfortunately, the half-life of the β_{1C} protein could not be evaluated in LNCaP or PC-3 cells as it was not detectable by immunoblotting analysis. Controls were performed to ensure that the solvent itself caused no difference in β_{1C} or β_{1A} steady-state protein levels (Fig. 5*A*). In order to better investigate the post-translational regulation of β_1 integrin splice variants, pulse-chase experiments were performed and the stability of the newly-synthesized β_1 integrin proteins was analyzed as described under "Experimental Procedures." Fig. 6 shows the results of a typical pulse-chase experiment: the decay of β_{1C} and β_{1A} nascent proteins was logarithmic both in normal and carcinoma prostate cells, and β_{1C} protein half-life was ~13 h in normal cells and ~8 h in neoplastic cells, whereas β_{1A} protein half-life was ~55 h in normal cells and ~17 h in carcinoma cells. Thus, these results show that post-translational processes, at the level of protein degradation, are involved in the regulation of β_{1C} and β_{1A} protein expression in prostate carcinoma cells.

The pulse-chase experiments were also used to investigate the maturation of β_{1C} and β_{1A} integrins in both normal and cancer cells. Fig. 6 shows, at 12 h of the chasing period in PNT1A cells, a decrease in the electrophoretic mobility of the nascent β_{1C} protein, with a 100% shift from the ~126-kDa form to one of ~130 kDa. Such a 130-kDa form was gradually degraded during the chasing period, as shown by the slight increase in mobility. In PC-3 and LNCaP cells, the rate of maturation of the β_{1C} protein was higher than in PNT1A cells: at 6 h of the chasing period, about half of the nascent β_{1C} protein showed a lower electrophoretic mobility, with a shift from ~126 to ~138 kDa. The 138-kDa fo[illegible]disappeared almost completely (more than 90%) at 12 h [illegible]ing period with a shift to the 126-kDa form that [illegible]lly lost. As far as the β_{1A} protein is con[illegible]s, at 6 h of the chasing period, a[illegible]cent protein showed a lower [illegible]ift from ~126 to ~138 k[illegible]85% of the total β_{1A} [illegible], a gradual protein [illegible]se in the electro-[illegible]P cells, a delay [illegible] at 6 h of the [illegible], *versus* 50% [illegible]tic mobility [illegible] kDa. The [illegible]e total β_{1A} pro-[illegible]and LNCaP cells, the [illegible]ich started at 12 h, was [illegible]lectrophoretic mobility, occur-[illegible] rate higher than in PNT1A cells. [illegible]carcinoma cells maturation of the β_{1A} [illegible] at a rate lower than β_{1C} maturation, as, at [illegible]sing period, ~ 10% of the β_{1A} nascent protein was [illegible]cessed *versus* ~50% of the β_{1C} nascent protein. In [illegible]r to find out whether the changes in electrophoretic mobility during β_{1C} and β_{1A} integrin processing were due to *N*-glycosylation, β_{1C} and β_{1A} immunoprecipitates of PNT1A and PC-3 cells were digested with endoglycosidase F (Endo-F) before electrophoresis. Fig. 7 shows that, upon removal of the *N*-linked oligosaccharides by Endo-F digestion, the β_1 integrin core protein was similar during processing for β_{1C} and β_{1A} integrins both in normal and in neoplastic cells. Overall, these results show that changes in the maturation process of the β_{1C} and β_{1A} integrin proteins occur in carcinoma *versus* normal prostate cells.

Proteolysis Pathways Involved in the Degradation of β_{1C} and β_{1A} Proteins—In order to gain some insight into the post-translational mechanisms that regulate β_{1C} and β_{1A} protein levels, the occurrence of the lysosomal-, the calpain- and the ubiquitin-mediated proteolysis in the degradative processes of the two splice variants was investigated by incubating PNT1A and PC-3 cells for varying times (up to 12 h) with either leupeptin, a lysosomal inhibitor, or MG132, a proteasome inhibitor, or ALLN, a calpain inhibitor, and by measuring changes in β_{1C} and β_{1A} protein levels by immunoblotting (Fig. 8). Treatment of PC-3 cells with leupeptin resulted in induction of β_{1C} protein expression and in increased β_{1A} expression. After 12-h treatment, the β_{1C} protein levels resulted ~40–50% of

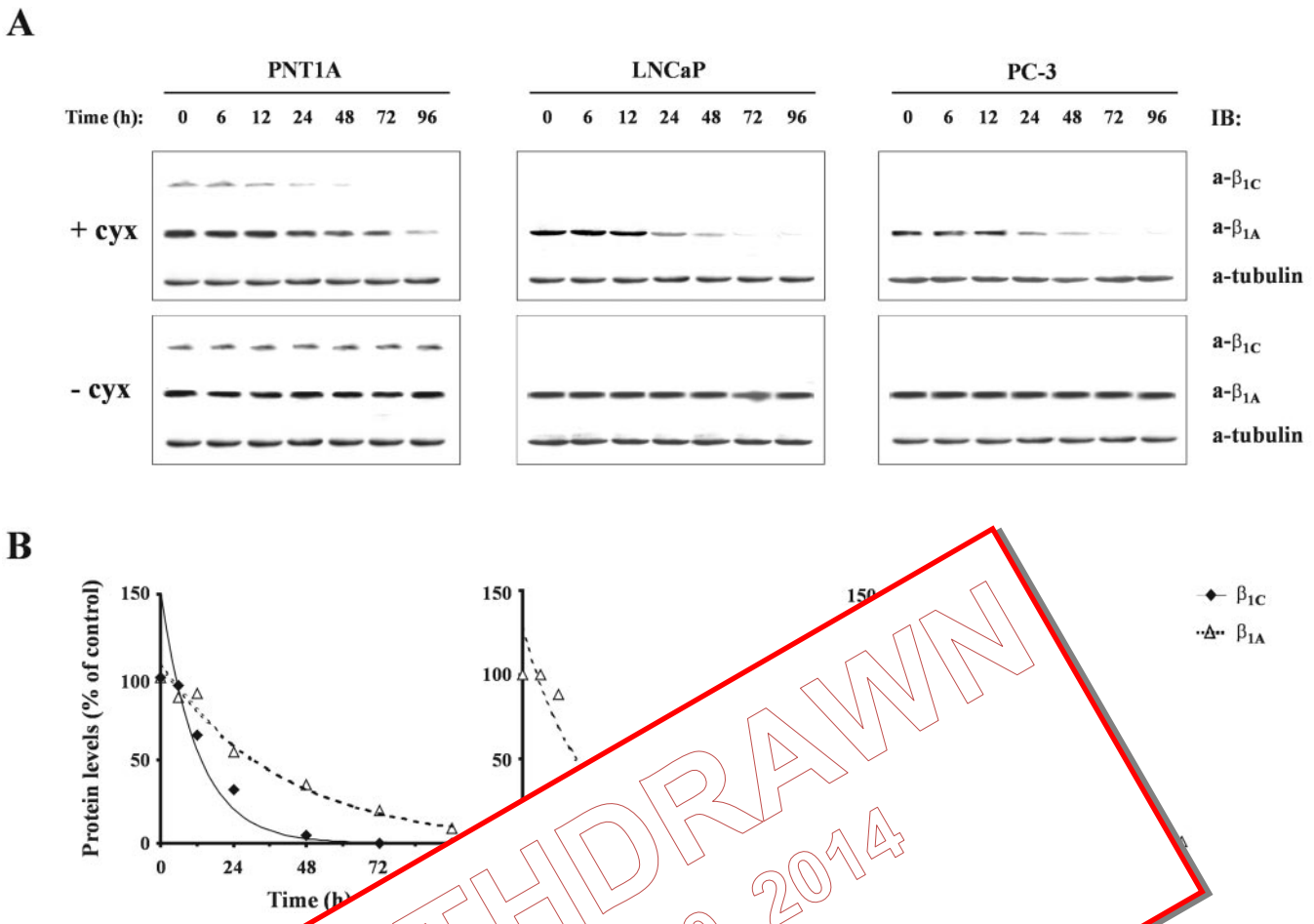


FIG. 5. **Stability of β_{1C} and** … were evaluated by addition of cycloheximide … were determined by immunoblotting analy… protein extracts (100 μg) were separated on 10% SDS… to either β_{1C} or β_{1A} integrin. β-tubulin was used to nor… β_{1C} and β_{1A} proteins in the presence of cycloheximide are repor… protein levels at time 0 (*control*), set at 100. Consistent results wer…

those expressed in normal c… normal but not in cancer cells. (*c*) The ubiquitin-increased of ~90–100% *versu*… some pathway degrades selectively the β_{1C} protein in results were obtained when th… prostate cancer cells.
or the lysosomotropic agent NH_4C… β_{1A}
Treatment of PC-3 cells with ALL… ted in a protein expression but treatment … affecting β_{1A} rapid induction of β_{1C} protein expres… expression. After 12-h treatment with …2, the β_{1C} protein levels resulted ~70–80% of those expressed in normal cells. As far as normal cells are concerned, inhibition of either the lysosomal- or the calpain-mediated proteolysis increased both β_{1C} and β_{1A} protein levels: leupeptin, as well as pepstatin and NH_4Cl (data not shown), caused an increase of ~ 45–50% both for β_{1C} and β_{1A} protein, whereas ALLN caused an increase of ~150% for β_{1C} and ~60–70% for β_{1A} protein after 12-h treatment. Neither β_{1C} nor β_{1A} protein levels were affected by MG132 treatment in normal prostate cells. Controls were performed to ensure that PNT1A and PC-3 cell viability, as judged by Trypan blue exclusion, was higher than 98% for all the treatments used (data not shown) and that incubation of normal and carcinoma cells with the solvent Me_2SO did not affect β_{1C} or β_{1A} protein levels. These results show the following. (*a*) The lysosomal proteolytic pathway is involved in the degradation of both β_{1C} and β_{1A} protein in normal and cancer prostate cells, albeit at a higher extent in cancer than in normal cells. (*b*) A calpain-mediated proteolysis of both β_{1C} and β_{1A} protein

If β_{1C} is a preferential target of the ubiquitin-proteasome pathway in cancer cells, inhibition of the proteasome activity with MG132 should accumulate ubiquitinated form/s of β_{1C} protein. To investigate this possibility, protein extracts of PC-3 cells treated with MG132 were immunoprecipitated with an antibody to either β_{1C} or β_{1A}, followed by immunoblotting analysis using an antibody to ubiquitin (Fig. 9). Upon treatment with MG132, ubiquitinated β_{1C} protein, migrating around 135 kDa, progressively accumulated as a function of time. As far as β_{1A} protein is concerned, two faint bands were observed 12 h after treatment but not in the earlier times. Of the two bands, one migrating around 135 kDa and the other around 105 kDa, the latter could represent an immature, non-glycosylated, β_{1A} protein form. As control of the total amount of β_1 integrins that were immunoprecipitated, immunoblotting was performed using an antibody that recognizes the extracellular domain of the β_1 integrins and, thus, all of the β_1 cytoplasmic variants. As shown in Fig. 9, the total amount of β_{1A} protein was significantly higher (at least 10-fold) than that of β_{1C} and did not change during MG132 treatment, at variance with β_{1C} protein levels that increased as a function of time (see also Fig. 8). Thus, in consideration of the much higher levels of β_{1A} *versus* β_{1C} protein, the amount of ubiquitinated β_{1A} protein, detected

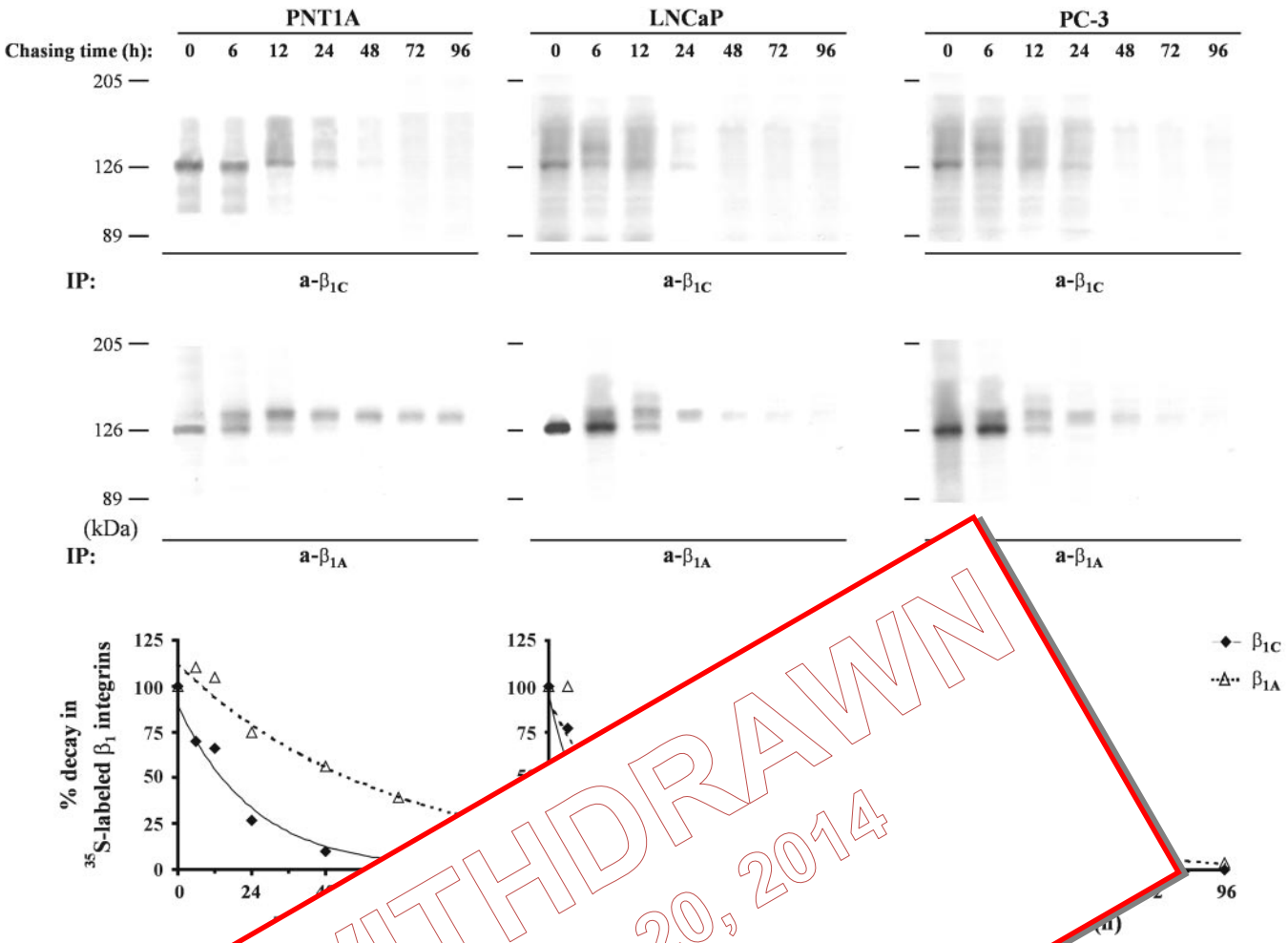


FIG. 6. **Stability an** ...beled with [^{35}S]methionine/cysteine (100 μCi/ml) fo... ...iods before being lysed. β_{1C} and β_{1A} proteins were immu... ...grin, electrophoresed on 10% SDS-polyacrylamide gel under ... ...s standards (kDa) are indicated. *Bottom*, a quantification of β_{1C} and ... ...ted as percentage of the protein levels at 0 h chasing period (*control*), set a... ...graphic signals of both the 126-kDa form and the forms with lower electrophoret... ...rom two independent experiments.

at 12 h, resulted extremely lo... ...These results, together with the findings already reported Overall, these results show that... ...prostate tissue (18, 19), represented the starting background teolytic pathway is involved in th... ...to further investigate the regulation of β_{1C} and β_{1A} expression β_{1C} protein in prostate carcinoma ... in prostate cancer.

DISCUSSION

We have investigated the molecular ...s involved in the regulation of β_{1C} and β_{1A} expression in normal and in cancer prostate cells. Indeed, the knowledge of the regulation of the expression of β_1 integrin variants in different pathophysiological conditions is rather poor; although it has been shown that β_1 integrin expression can be regulated both at the transcriptional (22–26, 30, 39) and post-transcriptional levels (23,25,27–31,40), these studies were performed on the β_1 integrin as a whole, with no discrimination among the various splice variants. We show here that, when comparing carcinoma *versus* normal prostate cells, β_{1C} and β_{1A} integrin variants share the same regulation at the transcriptional and post-transcriptional level, whereas they are differently regulated at the translational and post-translational level.

We show that, although in the investigated cancer cell lines LNCaP and PC-3 β_{1C} and β_{1A} mRNAs are expressed at lower levels than in the normal cell line PNT1A, the β_{1A} protein is expressed at comparable levels in LNCaP and PC-3 cells and in normal cells, but the β_{1C} protein is selectively lost in cancer

Changes in the transcriptional rate of the β_1 integrin gene have been previously shown to play a role in the regulation of β_1 integrin expression in cancer cells (26). A decrease in the transcriptional activity of the β_1 integrin gene has been recently demonstrated in human prostate cancer tissue (22). We show that a similar reduction also occurs in cancer cell lines, thus substantiating the validity of our model system. Moreover, we show that the stability of both β_{1C} and β_{1A} transcripts does not change during prostate malignant transformation, even though they differ from each other, the β_{1C} transcript half-life being much shorter than that of β_{1A} (~12 h *versus* $t_{1/2} > 48$ h) both in normal and cancer cells. Since β_{1C} and β_{1A} mRNAs differ only for a 116-nucleotide encoding sequence, absent in the β_{1A} transcript (14), we suppose that such a region is responsible for the lower stability. The short β_{1C} mRNA half-life could be considered when discussing the lower mRNA expression levels of this variant compared with β_{1A}, as here reported in prostate cells as well as in other cell types (14, 41).

As the β_{1A} integrin supports cell proliferation (13, 15) and cancer cell invasion (42), it is conceivable that high levels of

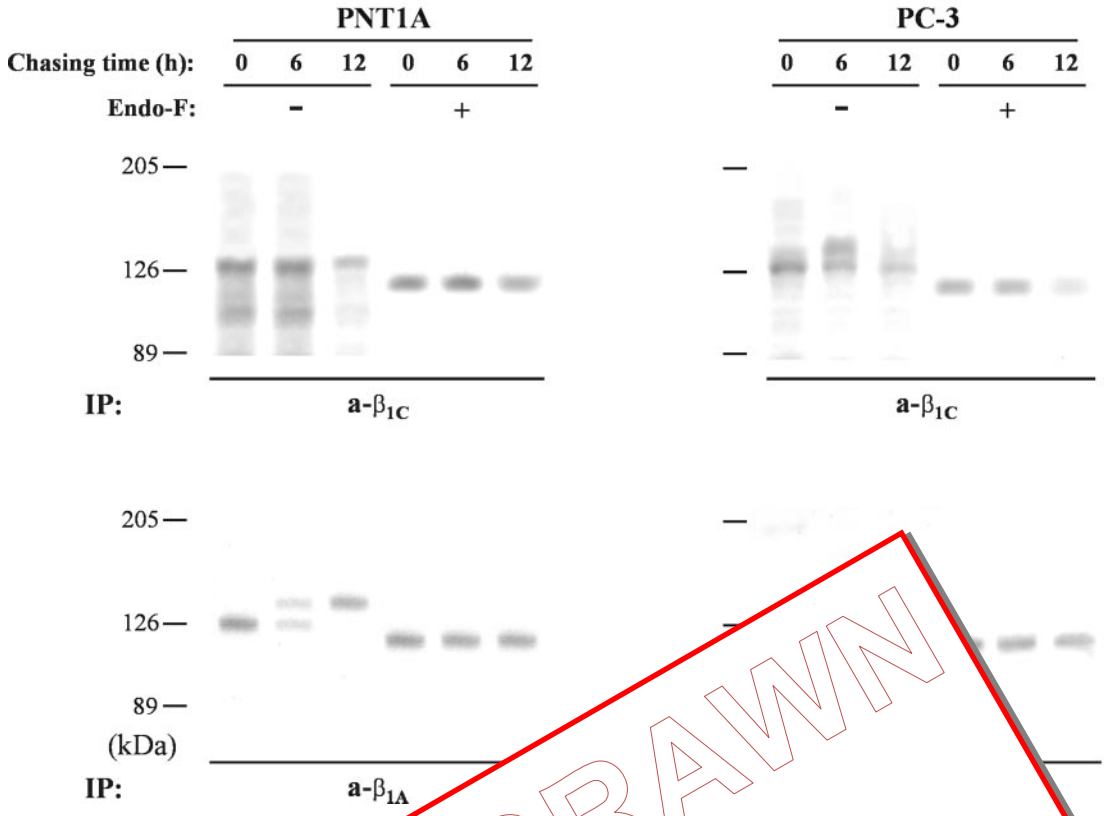


FIG. 7. **Sensitivity of β_{1C} and β_{1A} proteins to end** [^{35}S]methionine/cysteine (100 μCi/ml) for 3 h, then incubated in th re being lysed. β_{1C} and β_{1A} proteins were immunoprecipitate mmunoprecipitates were treated with (+) or without (− detected by fluorography. Positions of molecular xperiments.

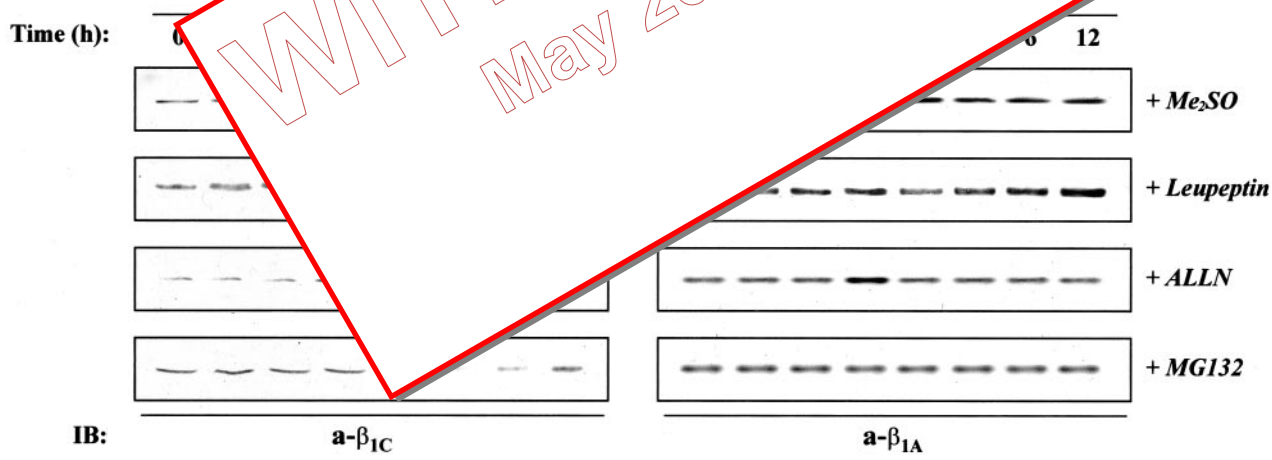


FIG. 8. **Sensitivity of β_{1C} and β_{1A} proteins to lysosomal, calpain, and ubiquitin proteolytic pathways.** PNT1A and PC-3 cells were treated with either leupeptin (100 μM) or ALLN (10 μM) or MG132 (10 μM) or the vehicle alone (Me_2SO) for the indicated times. Cell lysates were analyzed for the levels of β_{1C} and β_{1A} proteins by immunoblotting, as described in the legend to Fig. 5. Similar results were obtained from two independent experiments.

this protein are required in prostate cancer, even though the transcriptional activity of the β_1 integrin gene decreases. Thus, modifications in the translational/post-translational machinery in prostate cancer cells are expected to regulate β_{1C} and β_{1A} protein translation and/or degradation in an opposite manner, making it possible that low levels of β_{1C} protein, which strongly inhibits cell proliferation, but high levels of the β_{1A} protein, which favors cell proliferation and invasion, are expressed in prostate cancer.

In agreement with the above hypothesis, we show here that the β_{1C} translation rate decreases in prostate cancer cells, whereas the β_{1A} translation rate increases more than 2-fold *versus* the normal cells, notwithstanding the reduction in the mRNA levels. These results, in agreement with the general concept that deregulation of the protein synthesis machinery is a mechanism of neoplastic transformation, are consistent with several reports demonstrating constitutive high rates of protein synthesis for proteins having growth promoting properties (reviewed in Refs. 43 and 44).

A number of investigations have shown that cell glycosylation plays a significant role in malignant transformation (reviewed in Refs. 45 and 46) and can participate in integrin

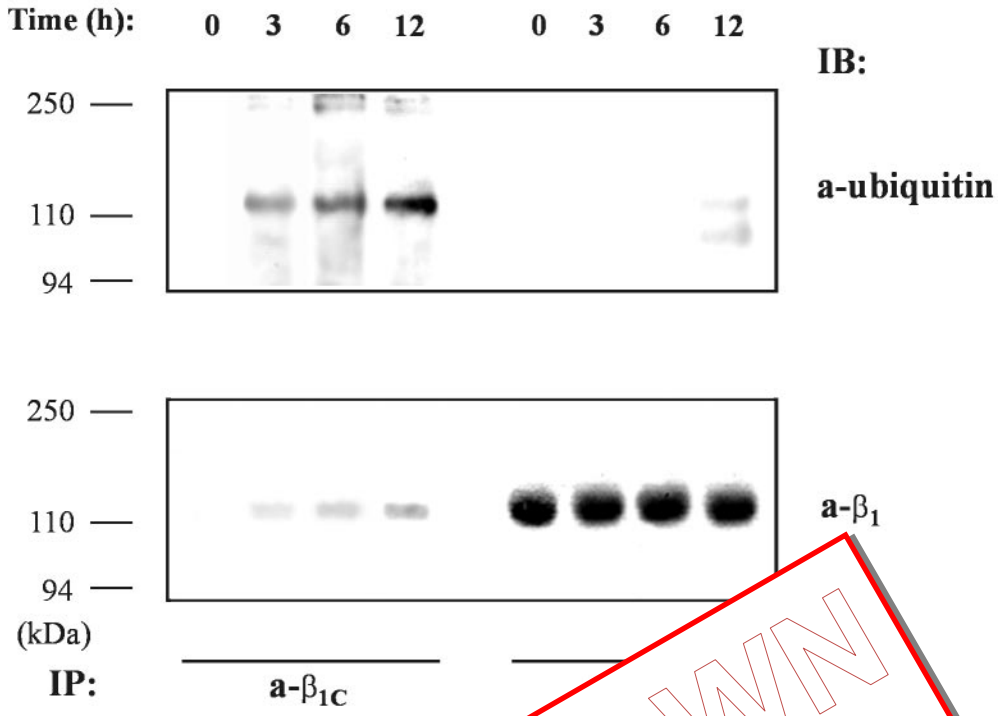


FIG. 9. **Ubiquitination of β_1 integrins in prostate cancer cells.** PC indicated times before being lysed. Cell lysates were first immunoprecipitated with an anti ted with an antibody to ubiquitin. Immunoblotting with an antibody to β_1 integri integrins that were immunoprecipitated. Positions of molecular mass standa two independent experiments.

protein degradation (47, 48). β_1 integri β_{1C} protein least twelve potential *N*-glycosylati in the extracellular domain (49) man cells, the addition of *N*-linked carboh 5). Degradation of occurs with concurrer shown to take place in (50). We show here with this finding, we show sized protein is glyco both β_{1C} and β_{1A} proteins occurs than in normal cells. In rostate cells, but that it is more active at 6 h of the chasing peri s, thus contributing to the loss of β_{1C} in (even if partially) in a fo ng the non-lysosomal pathways, the calpain higher than that observed a quitin-mediated proteolysis have been shown to be and since Endo-F treatment d in the degradation of many regulatory proteins (53, the shifts in electrophoretic mo , 59, 60). We show here that in normal, but not in cancer proteins, we suggest that inter prostate cells, the calpain-mediated proteolysis is involved in during processing may be differe cer the degradation of both β_{1C} and β_{1A} protein. It should be noted *versus* normal cells. We also show th rate of that many integrin subunits (including β_{1A}, β_{1D}, β_2, β_3, β_4, and the β_{1C} protein is different from that normal and β_7) have been shown to be calpain-sensitive (61–63). In particin cancer cells, as in PNT1A cells it is and in PC-3 and ular, cleavage of the β_{1A} cytoplasmic domain had been previLNCaP cells it is higher than that of β_{1A}. As aberrant *N*-ously demonstrated in normal cells (63). Our results show that glycosylation of β_1 integrins has been shown to affect cell ad- also the β_{1C} protein is a target of the calpain-mediated proteolysis (51) and migration (52), ascertaining whether the in normal cells. On the other hand, we show that in changes both in the processing rates and in the processing cancer cells, expression of β_{1C} protein, normally absent in these intermediates of the β_1 integrin variants depend on certain cells, is induced by inhibition of the ubiquitin proteolytic pathfeatures of the glycosylation could be a good goal to pursue. way, and reaches levels close to those expressed in normal cells, without any effect on β_{1A} protein levels, thus providing evi-

We show here that the β_{1C} protein is degraded more rapidly dence that the β_{1C} protein is a preferential target of the ubiqin cancer *versus* normal cells. However, we have found that the uitin-proteasome pathway in cancer cells. This is further conβ_{1A} protein half-life in cancer cells is also lower than in normal firmed by the preferential accumulation of ubiquitinated β_{1C} cells, suggesting that a general, non-splice variant-specific, and not β_{1A} protein when cancer cells are treated with a prohigher turnover of the β_1 proteins occurs in cancer cells. Such teasome inhibitor. We find that neither β_{1C} nor β_{1A} integrin is a conclusion is not unique: in fact, it has been already shown degraded by the ubiquitin-proteasome pathway in normal cells, that rapid degradation of normal proteins is a powerful, rapid indicating that the activity of the ubiquitin proteolysis, as well and specific means whereby key regulatory proteins, involved in as that of calpain, as discussed above, may vary between northe control of cell division, are irreversibly inactivated in cancer mal and carcinoma cells. These findings add another member, cells (reviewed in Refs. 53 and 54). In addition, we find that the *i.e.* the β_{1C} protein, to the list of growth inhibitory molecules β_{1C} protein half-life is significantly shorter than that of β_{1A} both that are preferentially degraded by the ubiquitin-proteasome in cancer and in normal cells, suggesting that the specific 48-

pathway in cancer cells (reviewed in Refs. 64 and 65) and that might be targeted by proteasome inhibitors in anticancer therapy (65, 66).

In conclusion, our data demonstrate that the differential expression of β_{1C} and β_{1A} integrins in prostate cancer cells is regulated at the transcriptional, post-transcriptional, translational and post-translational levels *via* both common (transcriptional) and variant-specific (post-transcriptional/translational/post-translational) mechanisms. Interestingly, we highlight a role for the ubiquitin-proteasome proteolytic pathway in the selective loss of β_{1C} protein expression in prostate cancer cells. Since *in vivo* down-regulation of β_{1C} seems to occur at an early stage in the pathogenesis of prostate cancer (18, 19), the identification of the specific molecular events that contribute to the loss of β_{1C}, but not β_{1A} protein in prostate cancer cells could help in giving some insights into the molecular basis of prostate malignant transformation.

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Regulation of β_{1C} and β_{1A} Integrin Expression in Prostate Carcinoma Cells

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