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β_1D Integrin Inhibits Cell Cycle Progression in Normal Myoblasts and Fibroblasts*

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Integrins are $\alpha\beta$ heterodimeric transmembrane receptors involved in the regulation of cell growth and differentiation. The β_1 integrin subunit is widely expressed *in vivo* and is represented by four alternatively spliced cytoplasmic domain isoforms. β_1D is a muscle-specific variant of β_1 integrin and a predominant β_1 isoform in striated muscles. In the present study we showed that expression of the exogenous β_1D integrin in C2C12 myoblasts and NIH 3T3 or REF 52 fibroblasts inhibited cell proliferation. Unlike the case of the common β_1A isoform, adhesion of β_1D-transfected C2C12 myoblasts specifically via the expressed integrin did not activate mitogen-activated protein kinases. The β_1D-induced growth inhibitory signal was shown to occur late in the G_1 phase of the cell cycle, before the G_1-S transition. Ha-(12R)Ras, but not (Δ22W)Raf-1 oncogene, was able to overcome completely the β_1D-triggered cell growth arrest in NIH 3T3 fibroblasts. Since perturbation of the β_1D amino acid sequence in β_1A/β_1D chimeric integrins decreased the growth inhibitory signal, the entire cytoplasmic domain of β_1D appeared to be important for this function. However, an interleukin-2 receptor-β_1D chimera containing the cytoplasmic domain of β_1D still efficiently inhibited cell growth, showing that the ectodomain and the ligand-binding site in β_1D were not required for the growth inhibitory signal. Together, our data showed a new specific function for the alternatively spliced β_1D integrin isoform. Since the onset of β_1D expression during myodifferentiation coincides with the timing of myoblast withdrawal from the cell cycle, the growth inhibitory properties of β_1D demonstrated in this study might reflect the major function for this integrin in commitment of differentiating skeletal muscle cells *in vivo*.

Integrins are a large family of heterodimeric transmembrane cell adhesion receptors (1). They are involved in many aspects of cell behavior and are known to regulate a number of intracellular signaling pathways (2–4). One of the key integrin functions is an adhesion-mediated growth signaling (5). Integrin-mediated positive cell growth signaling cascade leads to activation of MAP[1] kinases, which serves as a hallmark of cell proliferation (6–9). Integrins can synergize with growth factor receptors to relay the control of cell cycle progression in adhesion-dependent cell types (5, 10). Adhesion of different cell types to certain ECM proteins can either trigger proliferation or switch them to a differentiation program (11, 12). These opposite responses are possible due to redundant and overlapping expression of integrins and are mediated by ligation of different integrin $\alpha\beta$ heterodimers on the cell surface (1). Differential coupling of the Shc adapter protein to integrin α subunits causes selective activation of the MAP kinase cascade in adherent cells (13). This allows various integrin $\alpha\beta$ heterodimers to transduce distinct signals from the ECM to the cell interior, including positive and negative growth signals in response to adhesion.

β_1 integrin, the most ubiquitous β subunit, pairs with at least 10 different α subunits to comprise receptors for a wide variety of ECM proteins. It is abundantly expressed *in vivo* on all proliferating as well as differentiated growth-arrested cell types, excluding red blood cells (1). β_1 integrin is known to be involved in cell growth regulation in many cell types (14, 15). Four cytoplasmic domain variants generated by alternative splicing have been described for the β_1 subunit (Refs. 16–21 and reviewed in Ref. 22). In most cell types β_1 integrin is represented predominantly by the β_1A isoform. The only noticeable exception is in differentiated striated muscles where it is displaced by the β_1D integrin isoform (16). The other two minor β_1 integrin variants with the alternatively spliced cytoplasmic domains, β_1B and β_1C, were identified several years ago (18–21). Unlike β_1D integrin, these two isoforms are always coexpressed at low levels with the major β_1A isoform (18, 19, 21). Recently, the β_1C isoform has been shown to inhibit strongly cell growth upon transient expression in 10T1/2 fibroblasts (23). A short amino acid sequence Gln^{795}-Gln^{802} within the cytoplasmic domain is essential for β_1C-mediated growth arrest and is apparently unique for this integrin (24).

Striated muscle myoblasts become irreversibly withdrawn from the cell cycle early in myodifferentiation before cell fusion occurs. The timing of cell cycle withdrawal (commitment to myodifferentiation) coincides with the appearance of muscle-specific β_1D integrin in myocyte cultures and is in accordance with its increased expression in postmitotic growth-arrested myoblasts (16). We hypothesized that β_1D might transmit a growth inhibitory signal in normal myoblasts *in vivo*. Here we demonstrate an inhibition of cell proliferation by β_1D integrin when it is expressed transiently in normal myoblasts and fibroblasts.

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[1] The abbreviations used are: MAP, mitogen-activated protein; ECM, extracellular matrix; BrdUrd, bromodeoxyuridine; MBP, myelin basic protein; mAb, monoclonal antibody; IL2R, interleukin-2 receptor; DMEM, Dulbecco's modified Eagle's medium; CHO, Chinese hamster ovary; FBS, fetal bovine serum.

MATERIALS AND METHODS

Antibodies and Reagents—Thymidine analog bromodeoxyuridine (BrdUrd) and mouse anti-BrdUrd mAb were from Sigma. Mouse mAb TS2/16 against human β_1 integrin was provided by Dr. Martin Hemler (Dana Farber Cancer Institute, Boston) and used to detect the injected or transfected β_1A and β_1D integrins. Hamster anti-mouse β_1 mAb HMβ1-1 for visualization of the endogenous β_1 integrin was received from PharMingen (San Diego, CA). Anti-MAP kinase rabbit polyclonal antibody *sc-93* was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and was used for MAP kinase immunoprecipitation. Antibody against active (dually phosphorylated) MAP kinases was from Promega (Madison, WI). Rabbit polyclonal antibodies reacting with mouse cyclins A and E were obtained from Rockland (Gilbertsville, PA). Mouse mAb 7G7B6 against interleukin-2 receptor (IL2R) extracellular domain was from ATCC (Rockville, MD). Hamster mAbs reacting with mouse α_1, α_2, and α_5 integrins were obtained from PharMingen. Antibody against α_3 integrin cytoplasmic domain was kindly provided by Dr. Guido Tarone (University of Torino, Italy). Isoform-specific anti-β_1A and anti-β_1D antibodies were characterized previously (16).

Cell Cultures—Mouse C2C12 myoblasts and NIH 3T3 fibroblasts were obtained from ATCC and used between 3 and 10 passages. Ha-(12R)Ras- and (Δ22W)Raf-transfected NIH 3T3 fibroblasts were described previously (25–28). Briefly, activated Ha-(12R)Ras and activated (Δ22W)Raf-1 (25, 26) were cloned into the expression vector pZIP-NeoSV(×)1 and transfected into NIH 3T3 cells using the calcium phosphate precipitation technique essentially as reported previously (25, 27). 24 h after transfection G418 was added to the growth medium at 1 mg/ml. The cells were selected for 10 days by which time all the cells in a control mock-transfected dish were dead. NIH 3T3 cells stably expressing Ha-(12R)Ras or (Δ22W)Raf-1 displayed very similar highly transformed phenotype as detected by cell morphology, inhibited spreading, colony formation in soft agar, and profound cytoskeletal changes (26, 28). Rat embryo fibroblast line REF 52 was described previously (29). β_1A-CHO and β_1D-CHO stable transfectants were characterized previously (16, 17).

cDNA Constructs, Microinjection, and Transfection—The cDNAs for human β_1A and β_1D cytoplasmic domain isoforms in pECE vector were described previously (16, 19). cDNAs encoding β_1A/β_1D chimeras were prepared using the β_1A and β_1D cDNA fragments and polymerase chain reaction-based mutagenesis. The sequences of the four mutant β_1A/β_1D integrin cDNAs were verified by dideoxy termination sequencing. IL2R (interleukin 2 receptor) cDNA and IL2R-β_1A chimera (30) were kindly provided by Dr. Susan LaFlamme (Albany Medical College, Albany, NY). IL2R-β_1D chimera was generated using polymerase chain reaction amplification of the 3′ end of the β_1D cDNA (16) with the forward primer 5′-TGTAGCTGGTGTGGTTGCTG-3′ and the reverse primer 5′-TTCAAAGCTATTCTGGGCTG-3′. The polymerase chain reaction fragment was digested with *Hin*dIII endonuclease, and the resulting fragment encoding the β_1D cytoplasmic domain was inserted in the *Hin*dIII site of the IL2R-β_1A plasmid (30) to generate a plasmid encoding the IL2R-β_1D chimera. The structure of the IL2R-β_1D construct was confirmed by dideoxynucleotide sequence analysis using a primer upstream of the cloning site (5′-AGCGTCCTCCTCCTGAGTG-3′).

For nuclear microinjections the β_1A and β_1D integrin cDNAs were used at 0.5 mg/ml to inject C2C12 myoblasts, REF 52 fibroblasts, or NIH 3T3 cells growing on fibronectin-coated glass coverslips (23). Transfection of C2C12 myoblasts, REF 52 fibroblasts, and NIH 3T3 cells, either wild-type or expressing Ha-(12R)Ras or (Δ22W)Raf-1, was performed using Superfect™ Transfection Reagent (Qiagen, Santa Clarita, CA). Cells at 40–60% confluency were incubated with the transfection complexes in regular serum-containing growth medium for 3–5 h. 5 μg of DNA and 30 μl of Superfect™ was used for 60-mm dishes and 12 μg of DNA and 75 μl of Superfect™ for 100-mm dishes. Four independent transient transfections were performed for each set of experiments. As judged by immunofluorescence with TS2/16 mAb, 28 ± 9% C2C12 myoblasts, 19 ± 6% REF fibroblasts, and 31 ± 14% NIH 3T3 cells (either wild-type or transfected with activated Ha-Ras or Raf-1) expressed the exogenous human β_1A or β_1D integrins 24 h after transfection.

Integrin Analysis by Flow Cytometry—The expression levels of the transfected human β_1A and β_1D integrins were determined using fluorescence-activated cell sorter analysis with TS2/16 mAb which reacts identically with these two β_1 cytoplasmic domain variants (16, 17). The transfectants were incubated with 10 μg/ml of TS2/16 mAb for 1 h at 4 °C followed by fluorescein-labeled goat anti-mouse IgG. After the staining, cells were analyzed in a FACScan® flow cytometer (Becton Dickinson, Mountain View, CA).

Cell Proliferation Assay—The cell proliferation assay based on BrdUrd incorporation into the nuclei was described earlier (23, 31). Cells transfected with β_1A, β_1D cDNAs, β_1A/β_1D chimeras, or IL2R-β_1A and IL2R-β_1D constructs were trypsinized on the day after transfection and were plated on fibronectin-coated glass coverslips in DMEM containing 10% FBS. The next day, the growth medium was replaced for DMEM with 0.5% FBS and cells were kept in low serum for 24 h. After that, cells were switched to the medium containing 10% FBS and 50 μM BrdUrd. 24 h later, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline and permeabilized with 0.5% Triton X-100. Before the immunostaining, cells were incubated first with DNase (0.1 units/μl, Promega) for 30 min at 37 °C.

To determine which phase of the cell cycle is affected by β_1D integrin, NIH 3T3 cells on coverslips were starved in DMEM with 0.5% FBS for 2 days and then injected with β_1A or β_1D cDNA 0, 4, 8, 12, or 16 h after the addition of DMEM with 10% FBS. At least 250 cells were injected with each cDNA. More than 70% of the injected cells expressed the exogenous β_1A or β_1D proteins. In preliminary experiments, maximal protein expression was achieved 1–1.5 h after cDNA injection. 50 μM BrdUrd was added 18 h after the switch to 10% FBS, and the cells were incubated with BrdUrd for 6 h more. After BrdUrd treatment, cells were washed, fixed, and permeabilized as described above.

Immunostaining of the Transfected and Injected Cells—To visualize simultaneously the transfected (injected) β_1 integrins and nuclear incorporation of BrdUrd, fixed, permeabilized, and DNase-treated cells were coincubated with mAb TS2/16 against human β_1 integrins and anti-BrdUrd mAb (1:100 dilution) for 1 h at 37 °C. Rhodamine-labeled donkey anti-mouse IgG (Chemicon, Temecula, CA) was used as secondary antibody to visualize the expressed β_1 integrins at focal adhesions and accumulation of BrdUrd in the nuclei. Coverslips were viewed on a Zeiss Axiophot microscope equipped for epifluorescence. Micrographs were taken on T-max 400 film. 200 untransfected cells or cells expressing β_1A, β_1D, or β_1A/β_1D chimeras were counted and the nuclear staining for BrdUrd assessed.

BrdUrd-labeled C2C12 cells expressing IL2R-β_1A and IL2R-β_1D chimeric receptors were detected with mAb 7G7B6 against the extracellular domain of IL2 receptor (30) in combination with anti-BrdUrd mAb. 120 cells expressing IL2R construct, IL2R-β_1A, or IL2R-β_1D chimera were scored for the nuclear BrdUrd staining.

To detect nuclear localization of cyclins A and E in β_1A- and β_1D-transfected NIH 3T3 cells, they were initially treated as described above (see "Cell Proliferation Assay"). The cells were double-stained with mouse mAb TS2/16 and rabbit polyclonal antibodies against cyclins A and E, followed by a mixture of rhodamine-labeled donkey anti-mouse IgG and fluorescein-labeled goat anti-rabbit IgG (Chemicon). 120 β_1A- and β_1D-expressing NIH 3T3 cells were counted for both cyclin E and cyclin A expression.

In some experiments, intracellular localization of the endogenous β_1A integrin was examined in NIH 3T3 cells expressing human β_1D. In this case cells were incubated first with a mixture of mouse mAb TS2/16 against human β_1 integrin, mouse mAb against BrdUrd, and hamster mAb HMβ_1-1 specific for mouse β_1 integrin (PharMingen). This was followed by coincubation with rhodamine-conjugated donkey anti-mouse IgG and fluorescein-labeled donkey anti-hamster IgG (Chemicon).

MAP Kinase Assays—Activation of MAP kinases in β_1A- and β_1D-transfected C2C12 cells was studied as reported earlier (6, 9, 10, 16, 32, 33), with some modifications. Briefly, cells transfected with human β_1A and β_1D cDNAs were trypsinized on the day after transfection. The remaining trypsin was inhibited with 0.5 mg/ml soybean trypsin inhibitor, and cells were washed several times in serum-free medium. Cells in DMEM, containing 2% bovine serum albumin, were plated on bacterial Petri dishes, precoated with purified TS2/16 mAb against the transfected β_1 integrins, for 2 h at 37 °C. Unbound cells were washed out, and the adherent cells expressing human β_1A or β_1D integrins were lysed either in 1% SDS or in buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM HEPES, pH 7.5, with 1 mM sodium orthovanadate, 50 mM NaF, 1 mM *p*-nitrophenyl phosphate, 20 nM calyculin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Protein concentration in the samples was determined using Pierce BCA Protein Assay Reagent. Samples in 1% SDS (25 μg each) were used directly for SDS-polyacrylamide gel electrophoresis and subsequent immunoblotting with polyclonal antibody against activated (dually phosphorylated) MAP kinases (Promega). 0.5 mg of cell lysates in 1% Nonidet P-40, 0.5% sodium deoxycholate buffer were used for immunoprecipitation with *sc-93* anti-MAP kinase antibody (Santa Cruz Biotechnology) followed by immune kinase reaction with [γ-^{32}P]ATP and the exogenous substrate myelin basic

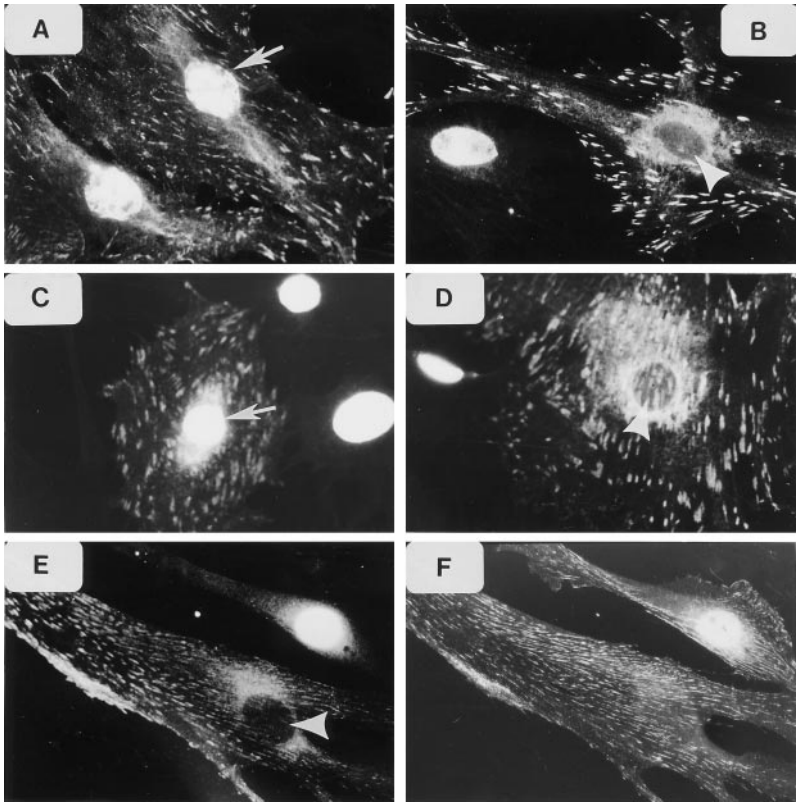

FIG. 1. **Expression of β_1D integrin in C2C12 myoblasts, REF 52, and NIH 3T3 fibroblasts inhibits proliferation.** *A–F*, effects of exogenous β_1A and β_1D expression on the nuclear incorporation of BrdUrd. *A* and *B*, C2C12 cells injected with human β_1A integrin (*A*) or human β_1D integrin (*B*). *C* and *D*, REF 52 cells injected with human β_1A integrin (*C*) or human β_1D integrin (*D*). *A--D*, cells were co-stained with anti-human β_1 integrin mAb TS2/16 and anti-BrdUrd mAb. *E* and *F*, β_1D-injected NIH 3T3 cells were double-stained for the expressed β_1D integrin and BrdUrd (*E*) and the endogenous mouse β_1A integrin (*F*). BrdUrd-positive nuclei in β_1A-expressing cells are marked by *arrows* (*A* and *C*). BrdUrd-negative nuclei in β_1D-expressing cells are indicated by *arrowheads* (*B*, *D*, and *E*).

protein (MBP). Phosphorylated MBP bands were analyzed by SDS electrophoresis and autoradiography (6, 16).

Metabolic Labeling, Immunoprecipitation, and Immunoblotting—Cultured NIH 3T3 fibroblasts (wild-type or expressing exogenous Ha-(12R)Ras) were metabolically labeled with 0.1 mCi/ml Tran^{35}S-label (ICN Biomedicals, Irvine, CA) in methionine- and cysteine-free medium for 12 h at 37 °C. After the labeling, cells were washed 3 times with phosphate-buffered saline and lysed on ice for 3 min with RIPA buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.5; containing 0.1% SDS, 1% Triton X-100 and 0.5% sodium deoxycholate). α_1, α_2, α_3, α_5, and the endogenous mouse β_1 integrins were immunoprecipitated using the specific antibodies against these subunits. The resulting immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis on 10% gels.

Cultured Ha-(12R)Ras-NIH 3T3 cells expressing human either β_1A or β_1D integrins were lysed in RIPA buffer and immunoprecipitated with the antibodies against the endogenous mouse α subunits or TS2/16 mAb against the transfected human β_1 integrins. The immunoprecipitates were extensively washed and boiled in SDS sample buffer. After SDS-polyacrylamide gel electrophoresis, β_1A and β_1D isoforms were detected in the immunoprecipitates by immunoblotting with the isoform-specific antibodies (16, 17).

RESULTS

In our preliminary experiments we were not able to obtain stable expression of β_1D in normal myoblasts, including mouse C2C12 cells or normal fibroblasts, such as REF 52 or NIH 3T3 cell lines, suggesting that this integrin might directly affect cell growth. To examine this possibility, we first microinjected either β_1A or β_1D cDNAs into the nuclei of these cells. The injected cells were serum-starved, then treated for 24 h with BrdUrd in serum-containing medium, and finally stained for both human β_1 integrin and BrdUrd incorporation (Fig. 1). No nuclear staining was seen in β_1A- and β_1D-injected cells with anti-β_1 integrin TS2/16 mAb alone (not shown). Whereas the majority of untransfected and β_1A-transfected cells displayed bright nuclear staining with anti-BrdUrd mAb (Fig. 1, *A* and *C*, *arrows*), most β_1D-transfected C2C12, REF 52, and NIH 3T3 cells did not incorporate BrdUrd into the nuclei (Fig. 1, *B*, *D*, and *E*, *arrowheads*). This indicated that β_1D caused growth arrest in these cell types. In the injected cells, β_1D colocalized with the endogenous β_1A integrin at focal adhesions but did not cause a displacement or relocalization of β_1A from these sites (Fig. 1, *E* and *F*). Therefore, we concluded that the observed inhibition of cell proliferation in β_1D-transfected cells was caused by the expression of β_1D integrin rather than the lack of the common β_1A isoform at cell-matrix contacts.

Transient transfection of β_1A and β_1D cDNAs in C2C12, REF 52, and NIH 3T3 cells resulted in similar expression levels of these integrins on the cell surface as determined by flow cytometry with TS2/16 mAb (Table I). Quantitation of the growth inhibitory effect mediated by β_1D in the transfectants showed that in the presence of this integrin only ~20% of C2C12 myoblasts (Fig. 2*A*) and ~14% of REF 52 fibroblasts (Fig. 2*B*) were entering the S phase, whereas more than 75% of β_1A transfectants were progressing into the cell cycle under conditions of this assay. Because the efficiency of cell growth arrest depends on the level of the exogenous β_1D, the few proliferating β_1D-transfected cells might represent a subpopulation of the transfectants with the lower level of β_1D expression. These results show that proliferation of normal myoblasts and fibroblasts is drastically inhibited by β_1D integrin.

Analysis of MAP kinase activation in β_1A- and β_1D-transfected C2C12 cells was first performed in the absence of exogenous growth factors in cells adherent to the substrate specifically via the expressed integrin (Fig. 3, *A* and *D*). β_1A-expressing cells displayed high levels of adhesion-dependent MAP kinase activation measured by both immunoblotting and immune complex kinase assays (Fig. 3, *A* and *D*; *a* and *a′*). In contrast, MAP kinase activity in the adherent β_1D transfectants did not differ from the basal levels characteristic for these cells in suspension (Fig. 3, *A* and *D*; *b* and *b′*). Unlike C2C12 cells expressing β_1D, β_1D-CHO transfectants exhibited sharp activation of MAP kinases in response to β_1D-mediated adhesion under the same experimental conditions (Fig. 3, *B* and *E* and Ref. 16). Therefore, the growth inhibitory effect of β_1D strongly depends on cellular context and might be suppressed

TABLE I
Surface expression levels of the exogenous human β_1A and β_1D integrins in the transfectants

Cell type	β_1A transfectants	β_1D transfectants
	mean fluorescence intensity[a]	
C2C12	460	370
REF 52	320	340
NIH 3T3	410	380
Ha-(12R)Ras-NIH 3T3	380	320
(Δ22W)Raf-NIH 3T3	340	350

[a] Values given are the means for four independent transfections as determined with TS2/16 mAb.

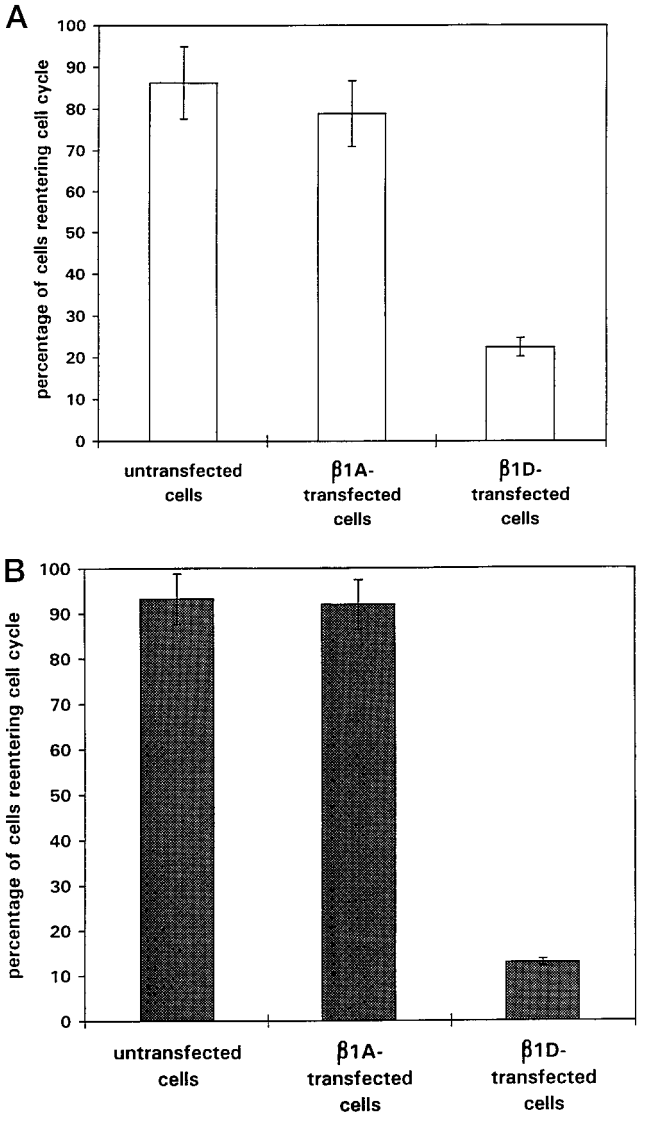

FIG. 2. **Quantitation of β_1D integrin-mediated inhibition of DNA synthesis in C2C12 myoblasts and REF 52 fibroblasts.** The percentages of C2C12 myoblasts (*A*) and REF 52 fibroblasts (*B*) showing nuclear staining with anti-BrdUrd mAb are depicted for untransfected cells, as well as for β_1A- and β_1D-transfected cells.

by enhanced activity of certain oncogenes.

In the presence of growth factors both β_1A- and β_1D-transfected C2C12 cells displayed higher levels of MAP kinase activation compared with serum-free conditions (Fig. 3, *C* and *F*). However, whereas MAP kinase activation further increased upon adhesion in β_1A-transfected cells (Fig. 3, *C* and *F*; *a* and *a′*), no such enhancement was seen in β_1D-expressing cells in the presence of serum (Fig. 3, *C* and *F*; *b* and *b′*). This observation showed that β_1D down-regulates MAP kinase activity in

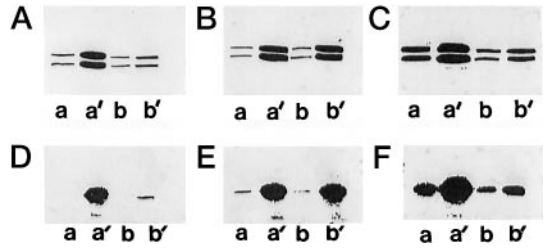

FIG. 3. **β_1D inhibits adhesion-dependent activation of MAP kinases in C2C12 myoblasts.** C2C12 myoblasts (*A*, *C*, *D*, and *F*) or CHO cells (*B* and *E*) expressing exogenous human β_1A (*a* and *a′*) or β_1D (*b* and *b′*) integrins were either kept in suspension (*a* and *b*) or plated for 2 h on plastic dishes coated with TS2/16 mAb against the expressed integrins (*a′* and *b′*). The experiments were performed either in serum-free medium (*A*, *B*, *D*, and *E*) or in 10% serum-containing medium (*C* and *F*). MAP kinase activation was measured by immunoblotting with antibody against dually phosphorylated MAP kinases (*A–C*) or by immune complex kinase assay with MBP as the exogenous substrate (*D–F*).

growth factor-stimulated C2C12 cells as well. Thus, the withdrawal of differentiating myoblasts from the cell cycle *in vivo* could be attributed at least partly to growth inhibitory effect of β_1D integrin.

To determine the phase of the cell cycle affected by β_1D expression, we double-stained both populations of NIH 3T3 transfectants for the expressed human β_1 integrin and either cyclin E or cyclin A (Fig. 4*A*). Scoring the β_1A- and β_1D-transfected cells for nuclear cyclin staining demonstrated that cyclin E expression did not significantly differ between the two populations. However, the percentage of β_1A transfectants expressing cyclin A exceeded drastically that of β_1D-expressing cells. Since the expression of cyclin A occurs specifically during the S phase and β_1D blocks its appearance in the transfected NIH 3T3 cells, we concluded that β_1D strongly interferes with the G_1-S transition. To analyze further the timing of the β_1D-triggered cell cycle block, we microinjected β_1D cDNA at certain time points after serum stimulation of starved cells (Fig. 4*B*). DNA synthesis in NIH 3T3 cells became insensitive to inhibition by β_1D integrin when its cDNA was injected ~12 h after serum stimulation. Previously, our estimations showed that the G_1 phase lasts ~14–16 h in this cell type. Together, these two observations demonstrated that β_1D-mediated growth arrest occurs late in the G_1 phase before the beginning of the S phase.

Previously we were able to select stable β_1D integrin transfectants of CHO cells (16) and GD25 β_1 integrin-deficient cells (17, 34), indicating that some cells were able to proliferate in the presence of β_1D. Since both these cell lines display elevated activity of certain oncogenes, we rationalized that some of them can overcome the β_1D-triggered cell cycle block. To test this suggestion directly, we transiently transfected β_1D cDNA into NIH 3T3 cells expressing constitutively activated forms of Ha-Ras and Raf-1 oncogenes (25–28). Analysis of cell proliferation by the BrdUrd incorporation assay demonstrated that Ha-(12R)Ras completely abolished the inhibitory effect of β_1D on cell growth (Fig. 5). However, (Δ22W)Raf-1 was not able even to diminish this β_1D-mediated effect. These data showed that certain oncogenes, such as Ha-Ras, overcome the β_1D-mediated growth arrest, whereas some others, like Raf-1, cannot.

Since integrin α subunits are known to modulate cell growth through differential activation of MAP kinases (13), we asked whether activated Ha-Ras could alter the expression pattern of α subunits in NIH 3T3 transfectants or their association with β_1D integrin (Fig. 6). We did not see any detectable changes in the pattern of α subunits associated with β_1 integrin in NIH 3T3 cells transfected with Ha-(12R)Ras compared with the wild-type cells (Fig. 6, *A* and *B*). α_3 and α_5 integrins were the

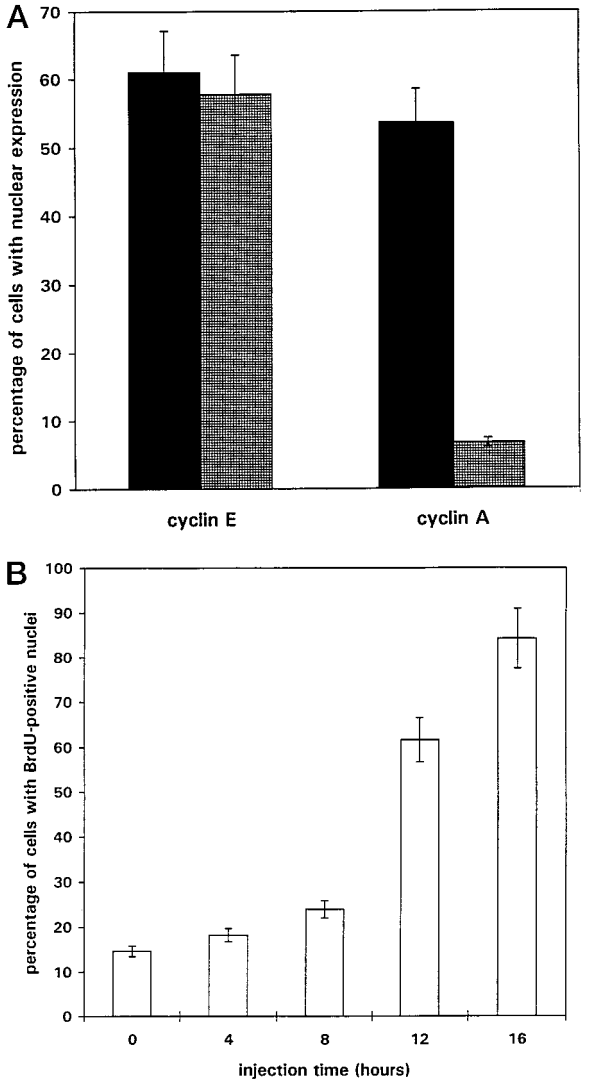

FIG. 4. **Determination of the cell cycle phase affected by β_1D integrin.** *A*, nuclear expression of cyclins E and A in β_1A- and β_1D-transfected NIH 3T3 cells. Cells were double-stained to detect the transfected β_1 integrin and cyclins E and A. Shown are the percentages of β_1A (*dark bars*) and β_1D (*hatched bars*) transfectants with the nuclear expression of cyclins E and A. *B*, quiescent NIH 3T3 cells were switched to serum-containing medium and microinjected with human β_1D cDNA at the indicated times thereafter. Cells were co-stained for β_1D expression and BrdUrd incorporation. The percentages of β_1D-NIH 3T3 cells with the nuclear BrdUrd expression are shown for each injection time point.

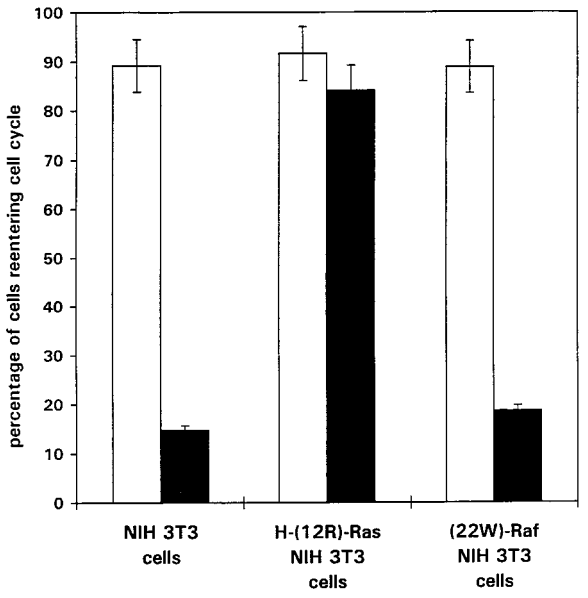

FIG. 5. **Activated Ha-Ras, but not Raf-1 oncogene, overcomes the growth inhibitory signal triggered by β_1D integrin expression.** NIH 3T3 cells, either wild-type or stably expressing Ha-(12R)Ras or (Δ22W)Raf-1 oncogenes, were transfected with human β_1A (*light bars*) or β_1D (*dark bars*) integrins. The percentages of β_1A- and β_1D-expressing cells incorporating BrdUrd in the nuclei were determined for each cell population.

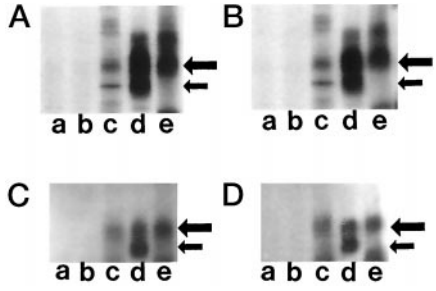

FIG. 6. **Expression of activated Ha-Ras in NIH 3T3 cells does not alter expression of α subunits or their association with the transfected β_1A and β_1D integrins.** *A* and *B*, immunoprecipitation of ^{35}S-labeled integrins from wild-type (*A*) or Ha-(12R)Ras-expressing (*B*) NIH 3T3 cells was performed with antibodies against mouse α1 (*a*), α2 (*b*), α3 (*c*), β_1 (*d*), or α5 (*e*) subunits. *C* and *D*, immunoprecipitates from β_1A-transfected (*C*) or β_1D-transfected (*D*) Ha-(12R)Ras-NIH 3T3 cells with antibodies against mouse α1 (*a*), α2 (*b*), α3 (*c*), α5 (*e*), or human exogenous β_1 (*d*) integrins were probed by immunoblotting with the isoform-specific antibodies against β_1A (*C*) or β_1D (*D*) integrins. *Long* and *short arrows* indicate the mature form and the precursor of the β_1 integrin subunit, respectively.

major β_1-associated α subunits in this cell line. The association of β_1A and β_1D integrins with the α subunits was not altered in the corresponding transfectants (Fig. 6, *C* and *D*).

We also wanted to examine which amino acid sequences in β_1D integrin are required and sufficient for its growth inhibitory function. We therefore created β_1 integrin chimeras by swapping β_1A and β_1D cytoplasmic domain sequences (Table II). All of these chimeric β_1A/β_1D integrins were expressed at similar levels on the cell surface and were targeted efficiently to focal adhesions of C2C12 cells, with the exception of chimera 4. None of the C2C12 transfectants expressing these chimeric integrins displayed abnormal adhesion or spreading (not shown). Interestingly, when examined by cell proliferation assay, none of the chimeras was as efficient as β_1D with regard to growth inhibition (Fig. 7*A*). The only chimera that still caused a significant decrease of C2C12 cell proliferation was chimera 2, which lacked only 6 amino acids at the very C terminus of the β_1D polypeptide (Fig. 7*A*, Table II). These data strongly suggest that there is no short amino acid motif within the β_1D cytoplasmic tail, which is sufficient for this function. Instead, the whole C-terminal half of the cytodomain, encoded by exon D of the β_1 integrin gene (16), appears to be essential for cell growth arrest.

By trying to determine whether the extracellular domain and ligand binding have a role in the growth inhibitory function of β_1D integrin, we took advantage of earlier engineered chimeric receptors, such as the IL2R-β_1A chimera (30). This chimera contains the extracellular and transmembrane domains of interleukin 2 receptor and the cytoplasmic domain of β_1A integrin. By replacing the β_1A portion of this construct with the cDNA fragment encoding the β_1D cytoplasmic domain (see "Materials and Methods"), we generated IL2R-β_1D chimeric receptor. Both IL2R-β_1D and IL2R-β_1A chimeras were similarly expressed on the cell surface (Table III) and were

TABLE II
Structure, surface expression levels, and focal adhesion localization of β_1A/β_1D integrin chimeras in C2C12 myoblasts

Construct	Amino acid sequence[a]	Mean fluorescence intensity[b]	Focal adhesion localization[c]
β_1A	AKWDT. . .GENPIYKSAVTTVVNPKYEGK	460	+++
β_1D	AKWDT. . .QENPIYKSPINNFKNPNYGRKAGL	370	+++
Chimera 1	AKWDT. . .GENPIYKSAVTTVVNPKYGRKAGL	390	+++
Chimera 2	AKWDT. . .QENPIYKSPINNFKNPNYEGK	310	+++
Chimera 3	AKWDT. . .GENPIYKSPINNFKNPKYEGK	430	++
Chimera 4	AKWDT. . .QENPIYKSAVTTVVNPNYGRKAGL	390	−

[a] Underlined are the amino acids derived from β_1D sequence.
[b] Values given are the means for three independent transfections as determined with TS2/16 mAb.
[c] Focal adhesion localization in C2C12 myoblasts was estimated as follows: +++, prominent localization at focal adhesion with little diffuse staining; ++, clear localization at focal adhesion with some diffuse staining; +, weak staining of focal adhesion with mostly diffuse localization; −, only diffuse staining.

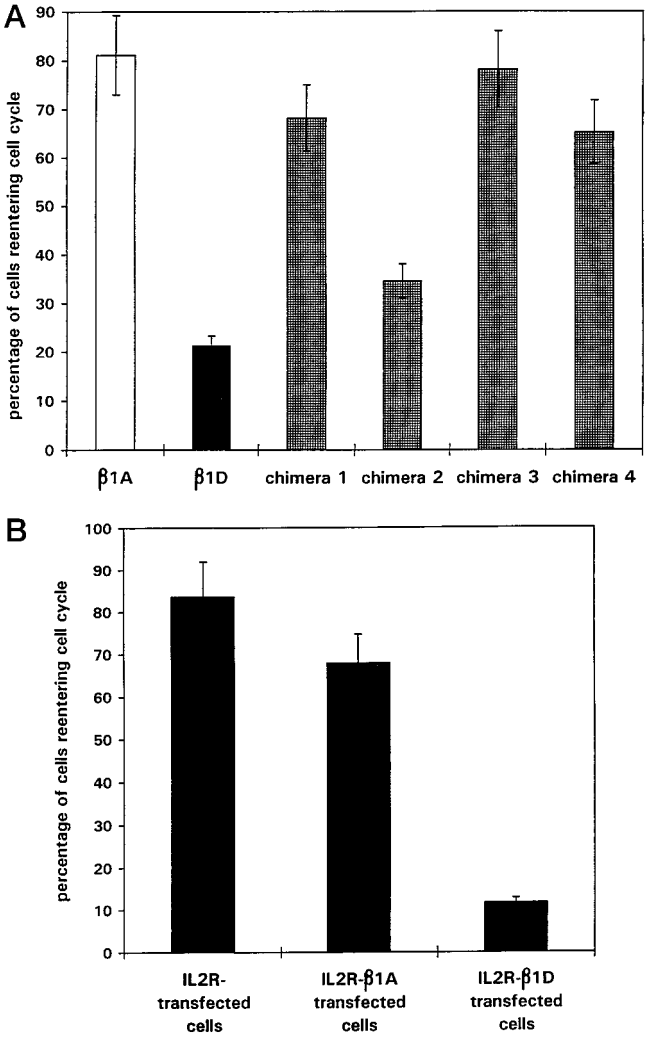

FIG. 7. **Analysis of the amino acid sequences required for the growth inhibitory effect of β_1D.** *A*, effects of swapping the cytoplasmic domain sequences on the β_1D integrin-mediated growth arrest. β_1A (*light bar*), β_1D (*dark bar*), and β_1A/β_1D chimeric integrins 1–4 (*hatched bars*; listed in Table II) were transfected in C2C12 myoblasts, and the percentages of proliferating cells in each population were determined by BrdUrd nuclear staining. *B*, the cytoplasmic domain of β_1D integrin is sufficient for the growth inhibitory signal. IL2R as well as IL2R-β_1A and IL2R-β_1D chimeric receptors were transiently expressed in C2C12 cells. Their effects on cell cycle progression was determined using the BrdUrd incorporation assay. Shown are the percentages of cells re-entering the cell cycle in all three cell populations.

targeted to focal adhesions upon transient expression in C2C12 cells (data not shown). The expression of these two chimeras as well as the wild-type IL2R in C2C12 myoblasts was followed by

TABLE III
Surface expression levels of IL2R-β_1A and IL2R-β_1D chimeric receptors in C2C12 myoblasts

Construct	IL2R	IL2R-β_1A	IL2R-β_1D
Mean fluorescence intensity[a]	680	490	440

[a] Values given are the means for three independent transfections as determined with 7G7B6 mAb against the extracellular domain of IL2R.

the analysis of cell proliferation using BrdUrd incorporation method (Fig. 7*B*). Our results showed that IL2R-β_1A chimera only slightly decreased proliferation, most likely due to partial disruption of focal adhesions in some of the transfectants expressing high levels of this construct. In contrast, IL2R-β_1D chimeric receptor strongly inhibited cell cycle progression in C2C12 myoblasts (Fig. 7*B*).

DISCUSSION

In this work we demonstrated that β_1D, the muscle-specific variant of the β_1 integrin subunit, inhibits cell cycle progression in normal myoblasts and fibroblasts. Our data reveal a novel function for the β_1D cytoplasmic domain isoform, which is the predominant β_1 integrin in striated muscles (16). This growth inhibitory function is specifically ascribable to the alternatively spliced cytoplasmic tail of β_1D, since the entire cytodomain appears to be both necessary and sufficient for the β_1D-mediated growth arrest.

β_1D exhibits a dominant-positive effect over the β_1A isoform due to enhanced interactions with both cytoskeletal and extracellular ligands and displaces the endogenous β_1A from cell-matrix contacts when expressed in certain cell types (17). However, in C2C12, REF 52 or NIH 3T3 cells expressing high levels of β_1D integrin, adhesion, and spreading on various ECM proteins was not visibly affected. Also, our recent data on transiently transfected β_1D-C2C12 and β_1D-NIH 3T3 cells indicated that even the cells expressing low amounts of β_1D and still having the endogenous β_1A at focal adhesions (Fig. 1, *E* and *F*) were unable to proliferate. These observations suggested that the β_1D-triggered cell cycle block is not caused by the lack of β_1A at cell-matrix adhesions and consequent loss of β_1A-mediated signaling but is primarily due to active signaling by β_1D. Meanwhile, further analysis is needed to elucidate whether this effect is based on the active inhibitory signal by β_1D or blocking positive adhesion-mediated signaling caused by this integrin variant.

Recently, another β_1 integrin isoform, β_1C, was shown to generate a strong growth inhibitory signal upon its transient expression in 10T1/2 fibroblasts (23) or CHO cells (24). Expression of this integrin *in vivo* is detectable in some blood cells (21) and is up-regulated in tumor necrosis factor-α-stimulated endothelial cells (24). No information is available yet concerning the mechanisms of growth arrest mediated by β_1D and β_1C isoforms. In both these cases, the cytoplasmic domains alone

are sufficient to generate the inhibitory signal, whereas the transmembrane and extracellular domains, and therefore ligand binding, are not required. However, in the case of β_1C integrin, a short specific motif Gln^{795}-Gln^{802} is responsible for this function (24), whereas the entire cytodomain appears to be important for the growth arrest triggered by β_1D. Another distinction between the mechanisms of growth inhibition by these integrins relates to the fact that β_1C displays its antiproliferative potential in all the cell types tested so far (23, 24), although β_1D growth inhibitory effect is clearly cell type-specific.

Certain oncogenes, as shown here for Ha-(12R)Ras, are able to completely suppress the β_1D-mediated growth arrest. This explains why we previously were able to obtain stable expression of β_1D integrin in some cell types that had elevated activity of some oncogenes (CHO and GD25 β_1-minus cells) but failed to select stable transfectants with other cells displaying a more normal phenotype. This property of β_1D is also consistent with the fact that some transformed cells of muscle origin, *e.g.* human rhabdomyosarcoma (RD) cells, proliferate even though expressing significant amounts of β_1D integrin.[2] Yet, others have shown that expression of c-*myc* and v-*myc* oncogenes in C2C12 myocytes does not significantly alter either their differentiation patterns or commitment (irreversible withdrawal from the cell cycle), (35). Taken together, these observations show that the growth inhibitory effect of β_1D can be suppressed by some but not all oncogenes.

At present it remains unclear why activated Ha-Ras is able to overcome the growth inhibitory signal from β_1D whereas activated Raf-1 can not. Most existing models of anchorage-dependent cell growth imply that Ras is positioned upstream from Raf-1 in the integrin-mediated MAP kinase cascade. Also, activated Raf-1 and Ha-Ras have similar transforming activity in NIH 3T3 fibroblasts as judged by cell morphology, growth in soft agar and cytoskeletal changes (26, 28). However, one recent report showed that integrin-mediated activation of MAP kinases, MEK (MAP kinase kinase) and Raf-1 in NIH 3T3 cells appeared to be independent of Ras (33). Also, activation of the Raf-1/MAP kinase cascade was shown to be insufficient for Ras transformation of RIE-1 epithelial cells (26). These recent observations imply that oncogenic Ras triggers certain Raf-independent signals essential for cellular transformation and also reevaluate the roles of Ras and Raf in propagation of adhesion-mediated growth signals from integrins to MAP kinases.

Previous analysis of β_1D expression in mouse myogenic cultures (16) and during mouse embryogenesis[3] showed that the onset of its biosynthesis coincides with the time point of irreversible growth arrest during myocyte differentiation. This indicates that the growth inhibitory properties of β_1D, demonstrated by transient transfection assays in this study, might be related to the major function of this muscle-specific integrin *in vivo*. Further work is apparently needed to elucidate the role of specific signaling events in the cell growth arrest mediated by β_1D integrin.

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