

# Inhibition of the degradation of the precursor and of the mature $\beta 1$ integrin subunit by different protein synthesis inhibitors and by ATP depletion

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A stabilization of both the precursor and the mature  $\beta 1$  integrin subunit was observed in metabolically labeled human skin fibroblasts and Molt-4 T lymphocytes upon addition of protein synthesis inhibitors or by ATP depletion. Differential effects of protein synthesis inhibitors are reported since the slow degradation of the mature  $\beta 1$  subunit was sensitive to cycloheximide but not to puromycin. We also show that the half-life of the mature subunit was not dependent on intracellular lysosomal degradation or on ubiquitination suggesting that VLA turn-over occurs at the cell surface and might involve proteins or proteases with short half-life.

VLA degradation; Protein synthesis inhibitor; ATP depletion; Ubiquitin; Lysosome

## 1. INTRODUCTION

Integrins are non-covalently linked transmembrane heterodimers composed of a single  $\beta$  subunit which can associate with several different  $\alpha$  subunits. At present, at least fourteen  $\alpha$  and eight  $\beta$  subunits have been described which combine to form functionally distinct cell surface molecules [1].

In an attempt to better characterize early events in the biosynthesis, the processing and the turn-over of VLA or  $\beta 1$  integrins, we showed previously that immature (endo-H sensitive)  $\alpha$  and  $\beta 1$  subunits could be coprecipitated and that in different cell types the  $\beta 1$  precursor was proteolytically degraded in a pre-Golgi compartment, sensitive to temperature and phenanthroline [2,3].

In the present study, we report that cycloheximide and other protein synthesis inhibitors inhibit the rapid degradation of the  $\beta 1$  precursor, while the slow degradation of mature subunit is only inhibited by cycloheximide but not by puromycin.

We also show that the slow turn-over of mature VLA-receptors is apparently not sensitive to lysosomotropic agents or to ubiquitination but can be prolonged by ATP perturbors, suggesting that processing at the cell surface is the main fate of these receptors.

## 2. MATERIALS AND METHODS

### 2.1. Reagents

Endo- $\beta$ -N-acetylglucosaminidase-H (endo-H), leupeptin, soybean

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trypsin inhibitor (SBTI), phenylmethylsulfonylfluoride (PMSF), hygromycin B and 1-chloro-3-tosylamido-4-phenyl-2-butanone (TPCK) were purchased from Boehringer. Antipain, pepstatin, and one batch cycloheximide were supplied by Sigma. Sepharose CNBr was from Pharmacia, Triton X-100 and 6-aminocaproic acid (6-AHA) from Janssen Chemica, NaDodSO<sub>4</sub> (SDS) from Serva, deoxycholate (DOC), NH<sub>4</sub>Cl, trichloroacetic acid (TCA), NaF and NaN<sub>3</sub> from Merck, cycloheximide from Calbiochem, puromycin and iodoacetamide from Aldrich, 3-methyladenine from Fluka and aprotinin from Bayer.

### 2.2. Antibodies

mAb DH12 and mAb HP2/1 recognize respectively the  $\beta 1$  and the  $\alpha 4$  subunits of the VLA integrins [4,5]. A rabbit antiserum (221M) against ubiquitin from Biomed and a mouse anti-ubiquitin mAb (mAb 1510) from Chemicon were used.

### 2.3. Cells

Diploid human skin fibroblasts (NHF) were grown and processed as described [2].

Molt-4 cells (ATCC) were cultured in DMEF-12 without TES and HEPES with 10–15% fetal bovine serum (FBS) (Gibco).

### 2.4. Metabolic labeling

Cell layers were preincubated (30 min) in cysteine-free medium (Select Amine-kit, Gibco) and metabolically labeled (30 min) with 0.1 mCi/ml [<sup>35</sup>S]cysteine ( $\pm 1000$  Ci/mmol; NEN). Chase was performed in culture medium without Ultrosor. At the appropriate time the cells were washed 3 times with 10 ml Tris buffered saline, pH 7.2, containing protease inhibitors (PI) (3 mM EDTA, 1 mM iodoacetamide, 1  $\mu$ g/ml pepstatin and 100  $\mu$ g/ml aprotinin), and scraped in this buffer.

Molt-4 cells were preincubated (45 min) in cysteine-free RPMI 1640 medium, [<sup>35</sup>S]cysteine was added at 0.25 mCi/ml for 30 min. After one washing, cells were resuspended in culture medium. At the appropriate time, cells were washed with ice-cold Tris-NaCl buffer (Tris-HCl 20 mM pH 7.4, NaCl 150 mM) containing PI (leupeptin, antipain, pepstatin and chymostatin at 1  $\mu$ g/ml, SBTI at 20  $\mu$ g/ml and 1 mM PMSF) and stored at  $-20^{\circ}\text{C}$ . Molt-4 buffers, except if otherwise mentioned contain 1 mM CaCl<sub>2</sub>/MgCl<sub>2</sub> to favor  $\alpha 4\beta 1$  coprecipitation.

The cells were lysed in 1 ml ice-cold Dippa buffer (Tris/NaCl buffer

supplemented with 1% Triton X-100, 1% DOC and 0.1% SDS and PI), then sonicated (15 s) and centrifuged at 17,000 rpm for 30 min.

#### 2.5. Immunoprecipitation and electrophoresis

Extracted proteins were precleared at 4°C on aspecific polyclonal rabbit anti-mouse IgG (RAM) (Dako) immobilized on Sepharose 4B beads. Immunoprecipitations with mAb DH12 were performed with 50 µl Sepharose 4B beads loaded with 5 mg/ml mAb. For immunoprecipitations with mAb HP2/1, 75 µl of mAb-containing culture supernatants were mixed with precleared cell lysates. After 2 h, immune complexes were isolated with 50 µl Sepharose 4B beads loaded with 10 mg/ml RAM IgG. The resulting immunoprecipitates were washed 5 times with 1 ml ice-cold Dippa buffer, and applied (eventually after reduction with 1%  $\beta$ -mercaptoethanol) on SDS-PAGE (6–10%). Gels were processed as described [2]. Autoradiograms were scanned using a Personal Densitometer (Molecular Dynamics).

#### 2.6. Endoglycosidase-H treatment

The antigen loaded immunobeads were digested for 16 h at 37°C with 5 mU Endo-H in 50 µl of 0.1 M sodium phosphate buffer, pH 5.5, containing 100 mM 6-AHA, 32 µg/ml leupeptin, 4 µg/ml pepstatin, and 200 U/ml aprotinin.

#### 2.7. Protein synthesis inhibition

Cells were preincubated 1 h (or 2 h), transferred in cysteine-free medium containing the inhibitor for 45 min and then labeled for 30 min. After two washings with Tris/NaCl buffer containing PI, the cells were solubilized and trichloroacetic acid precipitations done on cell extracts. The given percentages are the mean of three measurements of TCA precipitable labeled proteins from treated compared to untreated control cells. Table I gives the concentration of the inhibitor and its effect on protein synthesis.

### 3. RESULTS AND DISCUSSION

When NHF were pulse labeled with radioactive cysteine for 30 min and chased in the presence of cycloheximide (2.5 µg/ml), more  $\beta$ 1 precursor but less mature  $\beta$ 1 subunit could be precipitated than in the controls. The limited amount of endoH resistant  $\beta$ 1 subunit also showed a longer half-life (Fig. 1, upper panel).

Molt-4 cells treated under similar conditions and chased with cycloheximide (10 µg/ml) (Fig. 1, lower panel) also showed a stabilization of the  $\beta$ 1 precursor,

but maturation was apparently unaffected. The mature form was much more stable during the cycloheximide-chase than in controls. Cycloheximide preparations from 3 different commercial sources had similar effects.

Protein synthesis was substantially inhibited (90%), after 1 h preincubation with 20 µg/ml puromycin (Table I).

When puromycin was added to the NHF chase medium (Fig. 2, upper panel) the degradation of the  $\beta$ 1 precursor was also significantly decreased. Almost no mature  $\beta$ 1 subunit was immunoprecipitated.

In the Molt-4 cells (Fig. 2, lower panel) a similar effect was observed but, in contrast to cycloheximide (5 µg/ml) which seemed to stabilize the mature  $\alpha$ 4 $\beta$ 1 complex, puromycin (20 µg/ml) did not increase the half-life of the endo-H resistant heterodimer. To ensure that the activity of puromycin was not decreased after several hours of chase, an additional 20 µg/ml puromycin was added to the chase medium after 7 h, without additional effect, however, on the half-life of the mature  $\alpha$ 4 $\beta$ 1 (data not shown).

Hygromycin B (250 µg/ml) treatment of NHF gave results similar to puromycin (data not shown). Molt-4 cells were not tested since hygromycin B inhibited protein synthesis in these cells by only 20% (Table I).

Our results suggest a double effect of cycloheximide on  $\beta$ 1 degradation; it interferes with the rapid degradation of the  $\beta$ 1 precursor and with the slow disappearance of the endo-H resistant mature form. At both levels, in presumably different compartments, cycloheximide seems to stabilize the  $\beta$ 1 subunit. Possibly, the synthesis of a labile factor, which tags these glycoproteins for degradation, or of unstable proteinases, characterized by short half-lives, is affected by these compounds.

While puromycin is thought to be a more potent protein synthesis inhibitor than cycloheximide, puromycin was unable to increase the half-life of the mature  $\beta$ 1 subunit.

In addition to their different mechanisms of protein synthesis inhibition during translation, differential effects of puromycin and cycloheximide have only been documented at the transcriptional level; for instance of the metallothionein (MT) gene [6]. Similar findings to the ones described here have, however, not been documented.

Differences were also observed between both cell systems regarding the amount of mature  $\beta$ 1 immunoprecipitated. Indeed, the amount of mature subunit immunoprecipitated from NHF was significantly reduced, in comparison with the control, while no significant alteration in the maturation process was observed in Molt-4.

An explanation for this observation would be that differences in the maturation pathways or in the vesicular transport do exist between both cell lines or that protein synthesis inhibition interferes with a mannosyl-

Table I

Compound	Concentration	% inhibition of protein synthesis**	
		NHF	Molt-4
Puromycin	5 µg/ml	45%	
	20 µg/ml	90%	90%
	50 µg/ml		92%
Hygromycin	5 µg/ml	25%	
	50 µg/ml	45%	7%
	250 µg/ml	85%	12%*
	500 µg/ml		20%*
Cycloheximide	2.5 µg/ml	88%	
	5 µg/ml	90%	90%
	10 µg/ml	92%	92%

\*2 h preincubation instead of 1 h.

\*\*Each value represents the mean of one experiment done in triplicate.

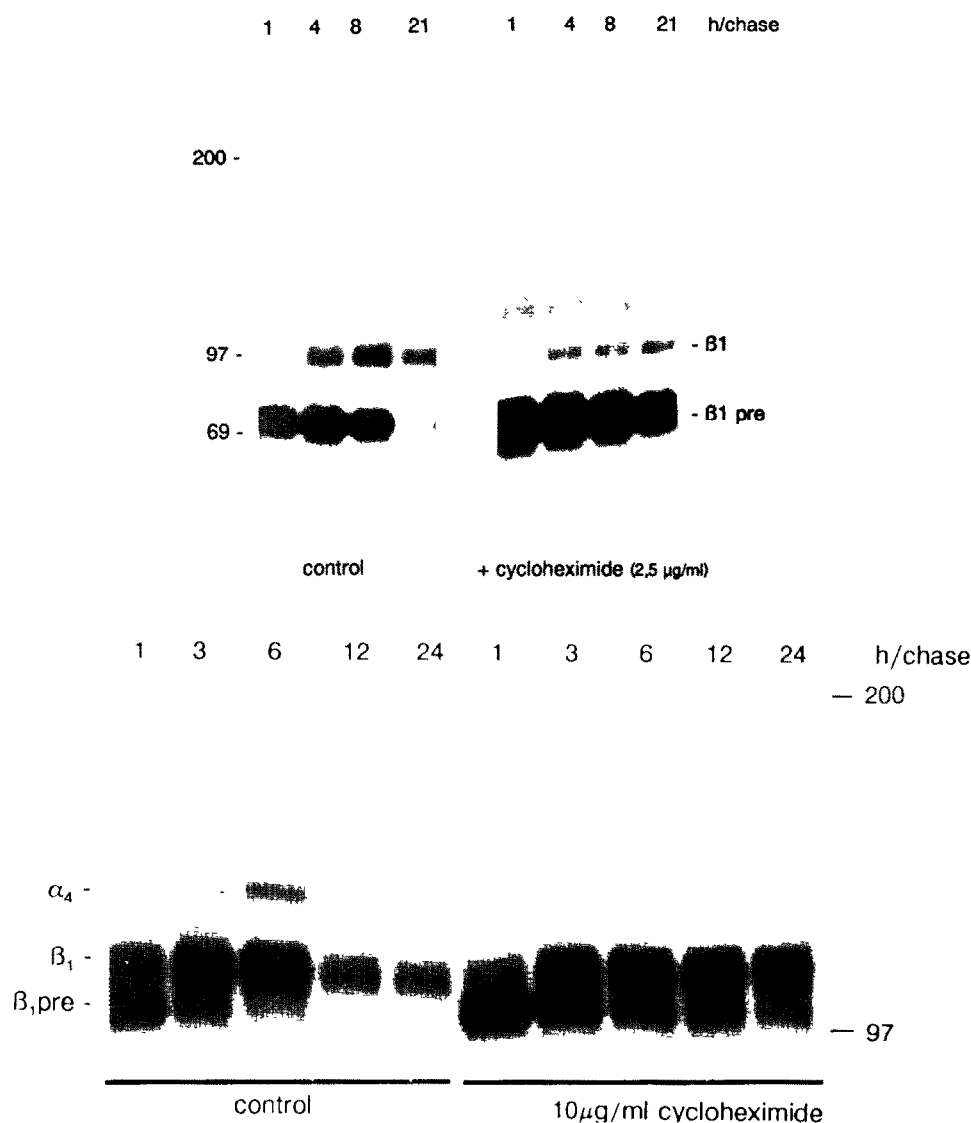


Fig. 1. (Upper panel) Effect of cycloheximide on the degradation of the  $\beta 1$  precursor in NHF. NHF cells were pulse labeled for 30 min with [ $^{35}$ S]cysteine and chased in complete medium for different times in the absence or presence of cycloheximide (2.5  $\mu$ g/ml). Cell lysates were immunoprecipitated with mAb DH12 and analyzed by SDS-PAGE after endo-H treatment.  $\beta 1$  pre is the precursor,  $\beta 1$  is the mature form. (Lower panel) Effect of cycloheximide on the synthesis and degradation of the  $\alpha 4$ - $\beta 1$  complex. Molt-4 cells were pulse labeled for 30 min with [ $^{35}$ S]cysteine and chased in the presence or absence of 10  $\mu$ g/ml cycloheximide for the indicated time periods. Cell lysates were immunoprecipitated with mAb DH12 and analyzed by SDS-PAGE under reducing conditions.

dase or transferase specifically involved in the trimming of oligosaccharides in NHF. Such effects on fibroblasts were nevertheless never reported previously.

Biosynthetic studies of  $\beta$ -N-acetyl-D-hexosaminidase A in fibroblast, have shown that cycloheximide inhibited the association and subsequent maturation of pre-formed  $\alpha$ -chains by inducing a depletion of the  $\beta$ -chain precursor pool upstream from the site of  $\alpha\beta$  association [7]. If cycloheximide induced a depletion of the  $\alpha$ -chain precursor pool, unassociated  $\beta 1$  integrin subunit would be unable to translocate out of the ER. Since the  $\alpha/\beta 1$  precursor ratio in Molt-4 is higher than in fibroblasts, quantitative differences between  $\alpha$  pools in both cell

systems could be responsible for the difference in the proportion of mature  $\beta 1$  observed.

The mature  $\beta 1$  subunit has a half-life of about 6 to 8 hours in Molt-4 (Fig. 3), and about 8 h in NHF [2].

Addition of a mixture of ATP synthesis inhibitors (NaF 5 mM/10 mM and  $\text{NaN}_3$  20 mM/40 mM) 7 h after the beginning of the chase partially blocked the degradation of the mature  $\alpha 4\beta 1$  dimer (between 15–30% in different quantitated experiments). Moreover, addition of 5 mM NaF and 20 mM  $\text{NaN}_3$  at the beginning of the chase stabilized the  $\beta 1$  precursor during, at least, the first 3 h of the chase (Fig. 4). These effects of cycloheximide and ATP depletion on the  $\beta 1$  precursor resem-

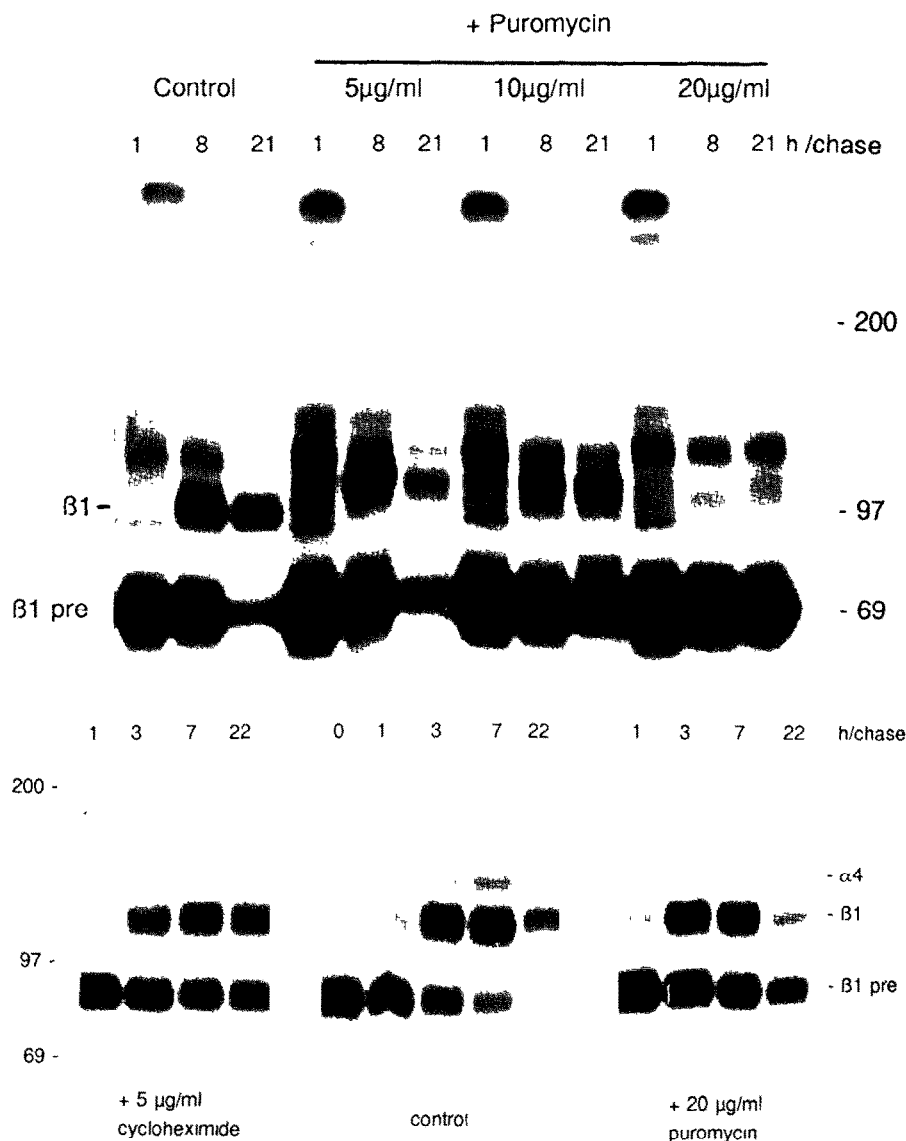


Fig. 2. (A) Effect of puromycin on the degradation of the  $\beta 1$  precursor in NHF. NHF cells were pulse labeled for 30 min with [ $^{35}\text{S}$ ]cysteine and chased in complete medium for different times in the absence or presence of different concentrations of puromycin. Cell lysates were immunoprecipitated with mAb DH12 and analyzed by SDS-PAGE after endo-H treatment. (B) Effect of puromycin on the synthesis and degradation of the  $\beta 1$  subunit in Molt-4. Molt-4 cells were pulse labeled for 30 min with [ $^{35}\text{S}$ ]cysteine and chased in the presence or absence of 20  $\mu\text{g/ml}$  puromycin for the indicated time periods. As control, cells chased in the presence of cycloheximide (5  $\mu\text{g/ml}$ ) are also shown. Cell lysates were immunoprecipitated with mAb DH12 and analyzed by SDS-PAGE under reducing conditions.

ble the stabilization observed under similar conditions of the 35 kDa intermediate fragment of unassembled H2a of the human asialoglycoprotein receptor in 3T3 fibroblast [8].

Addition of  $\text{NH}_4\text{Cl}$  or a thiol protease inhibitor (leupeptin) to the chase medium after 8 h of chase did not increase the half-life of the receptor suggesting that lysosomes or endocytosis were not involved in this degradation (Fig. 5) (for a review see [9]).

Recent evidence suggests that cell surface glycoproteins, such as the 90 kDa lymphocyte homing receptor recognized by MEL-14, can also be ubiquitinated [10,11]. Cell lysates of Molt-4 showed different ubiquiti-

nated proteins on Western blotting, but immunoprecipitates of  $\beta 1$  integrins with mAb DH12 did not reveal ubiquitinated forms of integrins. Moreover, 3-methyladenine, a specific inhibitor of autophagic protein degradation [12] had no effect on the stability of either the precursor or the mature  $\beta 1$  subunit (data not shown).

These results suggest that the half-life of the VLA-4 receptor in Molt-4 is not dependent on classic lysosomal or ubiquitination degradation pathways.

An alternative to intracellular degradation would be that  $\beta 1$  receptors disappear from the cell surface by shedding of entire membrane fragments (exosomes) as is the case for the transferrin receptor and MHCI-II

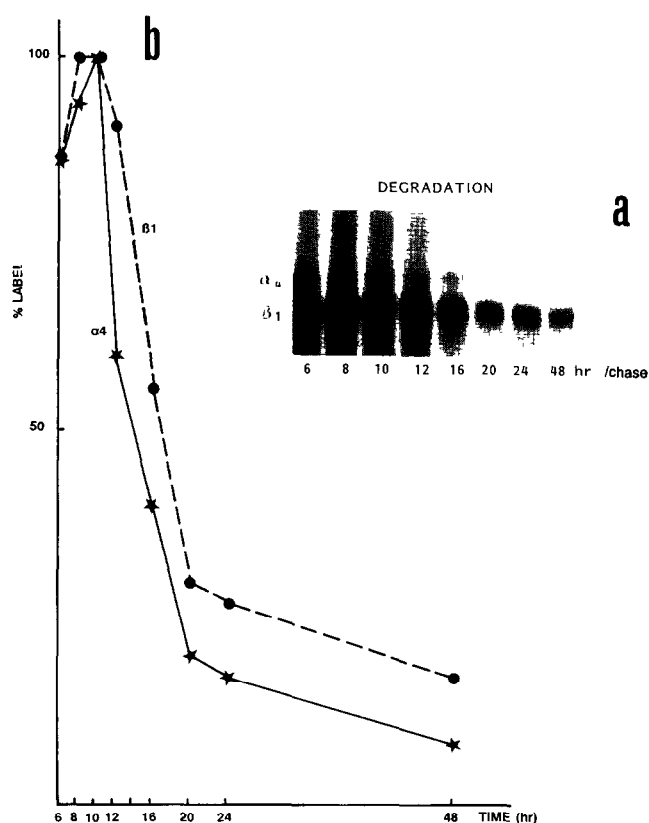


Fig. 3. Turn-over of VLA-4 on Molt-4. Molt-4 cells were pulse labeled for 30 min with [ $^{35}$ S]cysteine and chased for the indicated time periods. Cell lysates were immunoprecipitated with mAb DH12 and analyzed by SDS-PAGE under reducing conditions. Autoradiogram (a) was densitometrically scanned (b).

antigens, or by proteolytic degradation at the cell surface as is probably the case of the MEL-14 antigen which can be recovered from the medium as a 5–10 kDa shorter form upon addition of low doses of chymostatin [13,14]. In CHO cells, shedding of VLA-5 into the me-

dium upon binding with a specific mAb was reported by Sczekan and Juliano [15]. Cycloheximide then could act on the shedding or on receptor proteolysis.

A last possibility would be that the transport of mature endo-H resistant  $\alpha_4\beta_1$  complex from the *trans*-

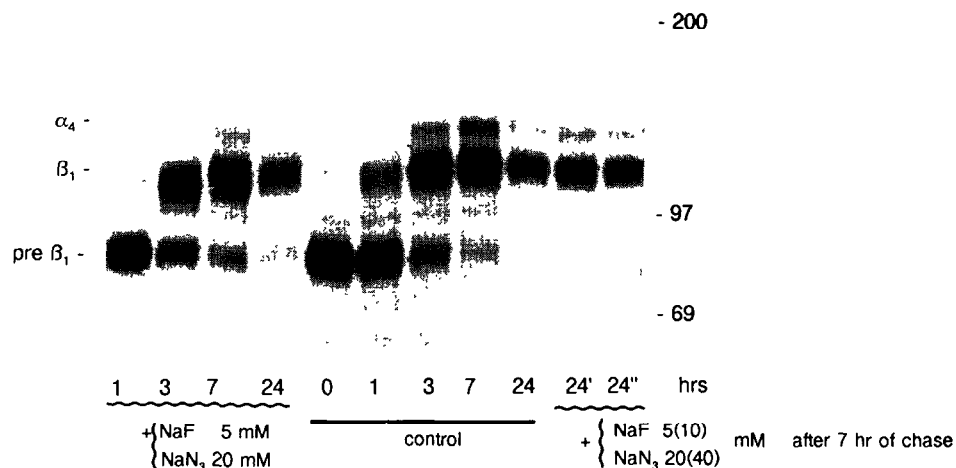


Fig. 4. Effect of ATP depletion on the degradation of  $\alpha_4\beta_1$ . Molt-4 cells were pulse labeled for 30 min with [ $^{35}$ S]cysteine and chased for the indicated time periods in the presence or absence of NaF (5 mM) and NaN<sub>3</sub> (20 mM). The 2 last lanes show samples chased 7 h before addition of (NaF 5 mM + NaN<sub>3</sub> 20 mM) (24') or (NaF 10 mM + NaN<sub>3</sub> 40 mM) (24''). Cell lysates were immunoprecipitated with mAb DH12 and analyzed by SDS-PAGE under reducing conditions.

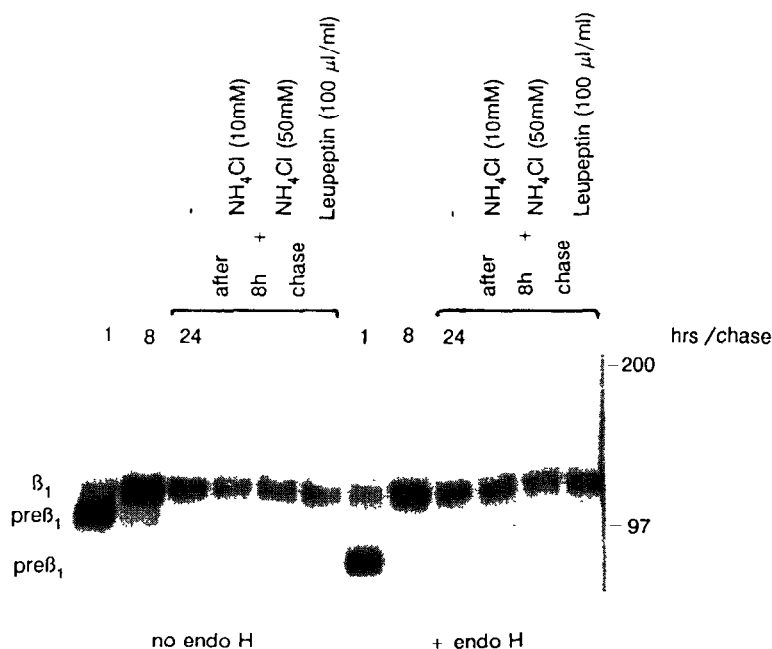


Fig. 5. Effect of lysosomal inhibitors on the degradation of the mature  $\beta 1$  subunit. Molt-4 cells were pulse labeled for 30 min with [ $^{35}$ S]cysteine and chased for 1 or 8 h. After 8 h of chase, cell aliquots were treated with lysosomal inhibitors (control without) and further chased up to 24 h. Cell lysates were immunoprecipitated with mAb DH12 in the presence of 5 mM EDTA in order to look only at the  $\beta 1$  subunit. Half of each sample was submitted to endo-H digestion and samples were analyzed by SDS-PAGE under reducing conditions.

Golgi to the surface is affected by cycloheximide, and that the complex is thereby stabilized.

Finally, since cycloheximide caused decreased coprecipitation of  $\alpha 4$  and  $\beta 1$  in Molt-4 cells a conformational change affecting their association cannot be excluded. Such a change could be due to an altered glycosylation (sialylation) for instance. It is interesting to note that treatment with cycloheximide abolished the adhesion of lymphoid cell lines to the heparin binding domain fragments of fibronectin [16]. How this observation relates to the ones described here remains to be determined.

In conclusion, the metabolism of VLA integrins seems to have a number of unusual properties, such as massive pre-Golgi degradation of the  $\beta 1$  precursor, sensitivity to protease inhibitors and to energy depletion, turn-over at the cell surface, which may be extremely important in the regulation of their expression and function.

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