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Stable expression of VLA-4 and increased maturation of the β_1-integrin precursor after transfection of CHO cells with α_{4m} cDNA

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Abstract A full-length cDNA coding for the murine α_4 integrin subunit (α_{4m}) was transfected into CHO-K1 cells and cell lines that expressed VLA-4 at their surface as a result of the association of transfected α_{4m} with endogenous hamster β_1 were selected. Functionality of the expressed $\alpha_{4m}\beta_1$ was shown by adhesion assays on VCAM-1 and antibody (anti-VCAM-1) inhibition. Pulse chase experiments indicated that transfection of the murine α_4 cDNA into CHO cells led to an increase in maturation and a decrease in degradation of the β_1 precursor subunit compared to control CHO-K1 cells. This was supported by FACS analysis, using an anti-hamster β_1 monoclonal antibody, which showed that more β_1 subunit was expressed at the surface of these stably transfected α_{4m} expressing cells. These results support the hypothesis that degradation of precursor β_1 is at least partly determined by the quantity of α subunits available intracellulary for heterodimer formation.

Key words: α_{4m} Integrin; Transfection in CHO; β_1 Metabolism

1. Introduction

Functional integrins are formed by the noncovalent association of an α subunit with a β subunit. To date, at least 14 α and 8 β subunits have been described giving rise to at least 20 distinct receptors [2–4]. In several reports concerning β_1 [5–7], β_2 [8] and β_3 integrins [9], β chains appeared to be synthesized in excess, suggesting that the availability of α subunits might be rate-limiting for the maturation of the β subunit. The reverse is observed, however, for the $\alpha_{IIb}\beta_3$ synthesis in megakaryocytes and human erythrocytic leukemia (HEL) cells [10,11] in which the α chain is synthesized in excess. Our previous work using Mab DH12 has shown that normal human skin fibroblasts degraded more than 75% of the β_1 precursor subunit before its maturation [7] and has provided evidence for a nonlysosomal pre-Golgi pathway for the degradation of the integrin β_1 precursor pool [12]. Others, using polyclonal antibodies, also observed a quantitatively important degradation of precursor β_1 subunit in human lung fibroblasts [5,6]. Akiyama et al. [13] demonstrated that the precursor pool of β_1 subunit in fibroblasts disappeared after malignant transformation of the cells. Such a pool of β_1 subunit also exists in CHO cells [14–16].

In this study, we compared the metabolism of the β_1 subunit in α_{4m} transfected and mock transfected CHO-K1 cells by pulse chase experiments. We found that the increase in α subunits synthesized in these cells resulted in an increased rate of β_1 subunit maturation, concomitant with an increase in functional β_1 subunit-heterodimers appearing at the cell surface.

2. Materials and methods

2.1. Plasmid and stable transfection

Plasmid pGEM3ZEB-$\alpha_4$37, containing a 3.7 kb full-length cDNA fragment of the murine α_4 subunit as described in our previous work [1], was *Eco*RI digested (an internal *Eco*RI site is present in the 3′ noncoding region of α_{4m}). The resulting 3.6 kb fragment was blunt cloned into the *Eco*RV site of the eukaryotic expression vector pcDNAI/Neo (Invitrogen) downstream of the CMV promoter (construct pcDNAI/Neo-$\alpha_4$36). About 10^7 CHO-K1 cells were electroporated at 250 V, 960 μF (Bio-Rad Gene pulser) with 40 μg of the pcDNAI/Neo-$\alpha_4$36 construct, the pcDNAI/Neo vector or H_2O as control. Forty-eight hours after transfection, the cells were placed in a 1:1 mixture of Ham F12/ Dulbecco's modified Eagles' medium (DME-F12) containing 10% FCS and 480 μg/ml G418 (geneticin, Gibco). Ten days later, when all the control cells died, limiting dilution was performed (20 to 40 cells per 96-well plate). G418 resistant cell lines were grown up and analysed by FACS or double immunoprecipitation for expression of α_{4m} using Mab R1/2 (anti-α_{4m}, PharMingen).

2.2. Cell–cell adhesion assays

About 40 μg human or mouse VCAM-1 cDNA in pCDM8 (kindly provided by Dr. R. Lobb, Biogen Inc., Cambridge, MA) was electroporated into 10^7 COS cells at 250 V, 960 μF, resuspended in DME-F12, 10% FCS and plated in 96-well plates at 75.000 cells/well. Adhesion assays were performed about 65 h post-transfection. The stably transfected CHO-K1 cell lines 3H12 (expressing α_{4m}) and 6A6 (negative control) were labeled overnight with 25–50 μCi/ml [^{35}S]cysteine (NEN). Single cell suspensions were prepared by washing once with versene, and incubation for 3 min in 0.05% twice-crystallised trypsin (Sigma). After one wash in complete medium and twice in DME-F12 containing 1% BSA the cells were passed through a double layer of 20 μm pore-size nylon filter and diluted to $2 \cdot 10^5$ cells/ml. For adhesion, 50 μl cell suspension (10^4 cells)/well was added to the washed COS microtiterplate, incubated at 37°C for 30 min and washed three times with DME-F12, 1% BSA. Monolayers and adherent labeled cells were dissolved in two changes of 1% SDS, 0.1% NaOH in H_2O, transferred to vials and counted by liquid scintillation. All assays were done in duplicate or triplicate and adhesion was expressed as the percent of the total radioactivity added per well. For antibody inhibition assays, transiently transfected COS plates were pre-incubated with blocking Mab 4B9 (anti-human VCAM-1, kindly provided by Dr. J. Harlan, University of Washington) at different concentrations (ascites dilutions between 1/200–1/10^4) for 30 min at 37°C before adhesion.

2.3. Pulse–chase experiments

Stably transfected CHO-K1 cell lines 3H12 (α_{4m} expressing) and 6A6 (control) were grown in DME-F12 supplemented with 10% FCS and G418 (480 μg/ml) in 75 cm^2 plastic flasks (T-75 flasks). About 65 h after trypsinization cell layers were preincubated for 30 min in cysteine-free medium (Select amino kit, Gibco) and than labeled for 30 min with 0.1 mCi/ml [^{35}S]cysteine (1000 Ci/mmol). After this, the cells were switched to complete medium supplemented with 2% Ultroser + G418 and chased for the appropriate time points. Cells were washed with ice cold Tris-NaCl buffer (Tris-HCl 20 mM pH 7.4, NaCl 150 mM) and scraped. Double immunoprecipitations were performed, by using the Mab 7E2 (anti-hamster β_1), as described previously for the Mab HP2/1 by Vekeman et al. [17]. Fluorography was performed as described by

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Bonner and Laskey [18] and dried gels were exposed to preflashed films to improve linearity of the obtained signal [19]. Autoradiograms were densitometrically scanned using a Personal Densitometer (Molecular Dynamics).

3. Results and discussion

A 3.6 kbp full-length cDNA fragment of the murine α_4 subunit [1] was cloned into the eukaryotic expression vector pcDNAI/Neo (Invitrogen) and transfected into CHO-K1 cells for stable transfection. G418 resistant cell lines were analysed by FACScan for surface expression of the murine α_4 subunit by using the Mab R1/2-FITC (anti-α_{4m}). One clone named 3H12 was clearly positive in FACS analysis compared to a control cell line stably transfected with pcDNAI/Neo (Fig. 1). A few other clones showed a smaller shift in fluorescence than the 3H12 clone. Double immunoprecipitations of ^{125}I surface labeled G418 resistant cell lines, by using the Mab R1/2, confirmed these results. Four bands of 150 kDa, 110 kDa, 80 and 70 kDa were obtained under reducing conditions (Fig. 2) in the α_{4m} expressing cell lines. These bands represent the mature α_{4m}, the β_1 (derived from the CHO-K1 cells and co-precipitating with α_{4m}) and the two smaller fragments of α_{4m} resulting from cleavage of the mature (150 kDa) α_{4m}, respectively. Genomic integration of the α_{4m} cDNA in the transfected CHO-K1 cell line 3H12 was confirmed by Southern-blotting (data not shown).

To determine whether the surface expressed murine α_4 subunit showed VLA-4 specificity, adhesion on COS-1 cells transiently transfected with human or mouse VCAM-1 was performed. These cells expressed VCAM-1 at their surface as determined by double immunoprecipitation using Mab 1G11B1 (anti-human VCAM-1,Monosan). Specific adhesion to VCAM-1 (about 50%) was obtained for the cell line 3H12, compared to the control 6A6 (Fig. 3). No adhesion was obtained on COS cells electroporated with water. The adhesion of the α_{4m} expressing cell line 3H12 to VCAM-1 transfected COS cells was inhibited by Mab 4B9 (30% of the specific binding) (Fig. 3).

In our previous work, we showed that human skin fibroblasts have an intracellular pool of β_1 precursor and that about 75% of the β_1 subunit precursor is degraded before maturation [7]. Other groups have shown that this pool of $\beta 1$ subunit also exists in CHO cells [14–16]. In addition, it has been suggested that the size of the intracellular pool and the rate of maturation of the β_1 precursor depended on the amount of α subunit available [6,13]. To examine the effect of transfection on the metabolism of the β_1 precursor, pulse chase experiments were performed on the stably transfected cell lines 3H12 (expressing α_{4m}) and 6A6 (negative control) (Fig. 4) using Mab 7E2 (anti-hamster β_1) [20]. The amount of β_1 precursor and mature β_1 subunit was determined. Densitometric scanning of the pulse chase experiments showed about 20% more mature β_1 subunit in the 3H12 cell line than in the control 6A6, concomitant with an apparent decrease in β_1 precursor degradation. Since the amount of mature β_1 subunit at each time point is expressed as the percent of β_1 precursor subunit present at time zero, and since the free [^{35}S]cysteine can be further incorporated during the first hours of chase, more than 100% mature β_1 subunit is obtained. Our results are in good agreement with the results of the FACS analysis using Mab 7E2 (anti-hamster β_1). A shift in fluorescence of about 20% was found between the control and the α_{4m} expressing CHO-K1 cell line (Fig. 1). Interestingly, this result appeared to be reversible. After three months in culture the cells gradually lost their expression of α_{4m}. This loss was accompanied by a decrease in surface expression of β_1, while pulse chase experiments revealed that the difference in β_1 metabolism between the 3H12 cells and the control cell line 6A6, gradually disappeared.

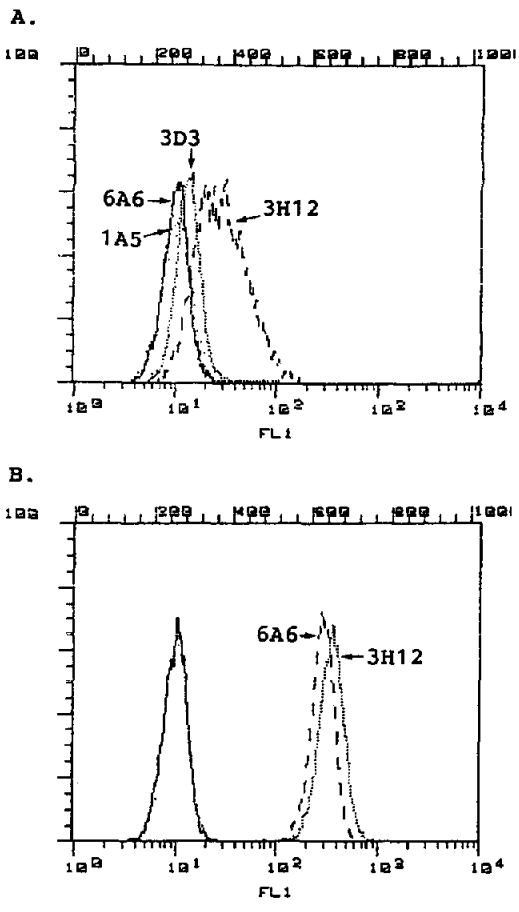

Fig. 1. Cell surface expression of α_{4m} and of β_1 on CHO-K1 transfectants. Flow cytometry of G418 resistant cell lines was carried out using (A) the fluorescein-labeled Mab R1/2 (anti-mouse α_4) and (B) Mab 7E2 (anti-hamster β_1) followed by fluorescein labeled goat-anti-mouse Ig. 6A6 is a control cell line transfected with the expression vector pcDNAI/Neo. 3H12, 3D3 and 1A5 are G418 resistant cell lines transfected with the pcDNAI/Neo-$\alpha_4$36 construct.

The absence of α subunits (under the detection limit) normally co-precipitating with the β_1 subunit in the presence of Ca^{2+}/Mg^{2+} might be due to the slower rate of synthesis of α subunits (α_5,α_{4m}) relative to β_1 and to the presence of a much lower amount of cysteine residues (about 50%) in the α subunit compared to the β subunit. Indeed, longer labeling periods or the use of TRAN^{35}S-label (ICN, [^{35}S]methionine/[^{35}S]cysteine) resulted in apparent co-precipitation (above the detection limit) of α subunits. This interpretation was supported by pulse–chase experiments performed using Mab R1/2 (anti-mouse α_4) instead of Mab 7E2 (anti-hamster β_1), in which at time zero no α_{4m} subunit was visible while after 3 h of chase, a strong band of

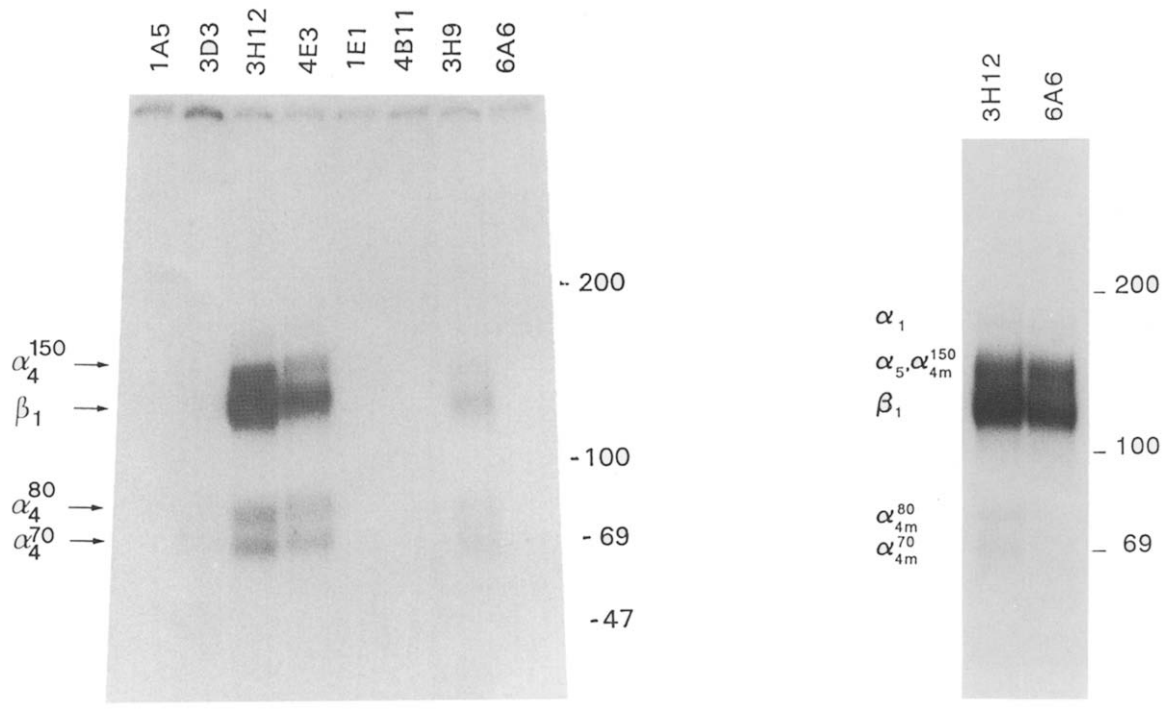

Fig. 2. (A) Cell surface expression of murine VLA-4 by CHO-K1 cells stably transfected with pcDNAI/Neo-$\alpha_4$36. Stable clones were selected expressing α_{4m} at their surface in association with β_1. Lysates of surface ^{125}I-labeled cells (lactoperoxidase method) were immunoprecipitated using Mab R1/2 (anti-mouse α_4). Immunoprecipitates were analysed by SDS-PAGE under reducing conditions. The positions of the different α_{4m} forms and β_1 are indicated. 6A6 is the negative control cell line. (B) Cell surface expression of β_1 integrins on cell line 3H12 (α_{4m} expressing) and 6A6 (control). Equal amounts of intact 3H12 and 6A6 cells were ^{125}I surface labeled by using the lactoperoxidase method. Lysates were immunoprecipitated by using Mab 7E2 (anti hamster β_1). Immunoprecipitates were analysed by SDS-PAGE under non-reducing conditions. The positions of the different α subunits and β_1 are indicated.

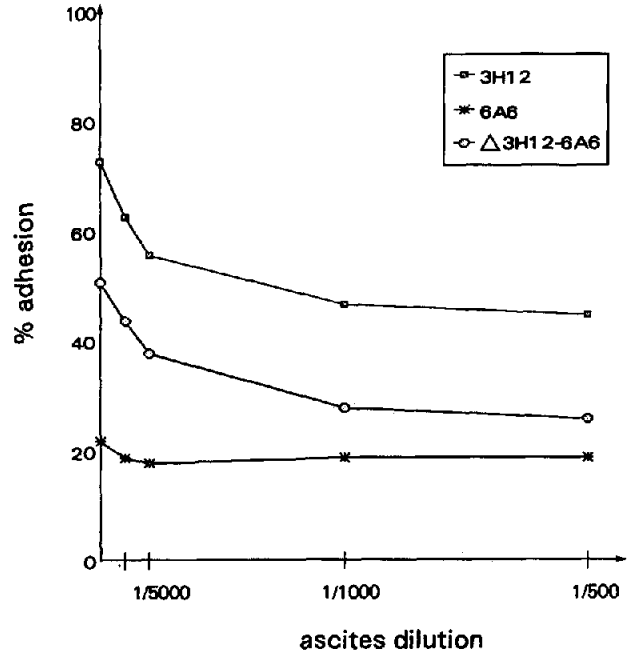

Fig. 3. Adhesion of α_{4m} transfectants to VCAM-1 expressing cell monolayers. Attachment of the stably transfected CHO-K1 cell lines 3H12 (α_{4m} expressing) or 6A6 (control) to COS1 cells transiently transfected with VCAM-1 and inhibition by the Mab 4B9 (anti-human VCAM-1) was performed as described in section 2. The inhibitory anti-VCAM-1 Mab 4B9 was added at the indicated ascites dilutions. Values are the means of an experiment done in triplicate and adhesion is expressed as the percent of the total radioactivity added per well. Δ3H12–6A6: difference in adhesion between the α_{4m} expressing cell line 3H12 and the control cell line 6A6.

mature β_1 subunit appeared to co-precipitate with the α_{4m} subunit (data not shown). To visualize the α chains complexed to the β_1 subunit in these cell lines, equal amounts of 3H12 cells and 6A6 control cells were surface labeled with ^{125}I and subjected to immunoprecipitation with Mab 7E2 (anti β_1 Mab). Fig. 5 shows a difference of about 30% in β_1 subunit expressed at the cell surface, while the only differences between the coprecipitating α subunits in these cell lines are the different forms of α_{4m}.

In this study we have shown that transfection of an α subunit into a cell which already contains β_1, makes additional β_1 available (from the precursor pool) for association with the new α, and that the level of the β_1 appearing at the cell surface increases. This result is in good accordance with the general model in which endo H sensitive α and β subunit assemble in the endoplasmic reticulum before being transported to the Golgi for further maturation and in which degradation of the excess β_1 precursor subunit occurs in the ER or a pre-Golgi compartment [12].

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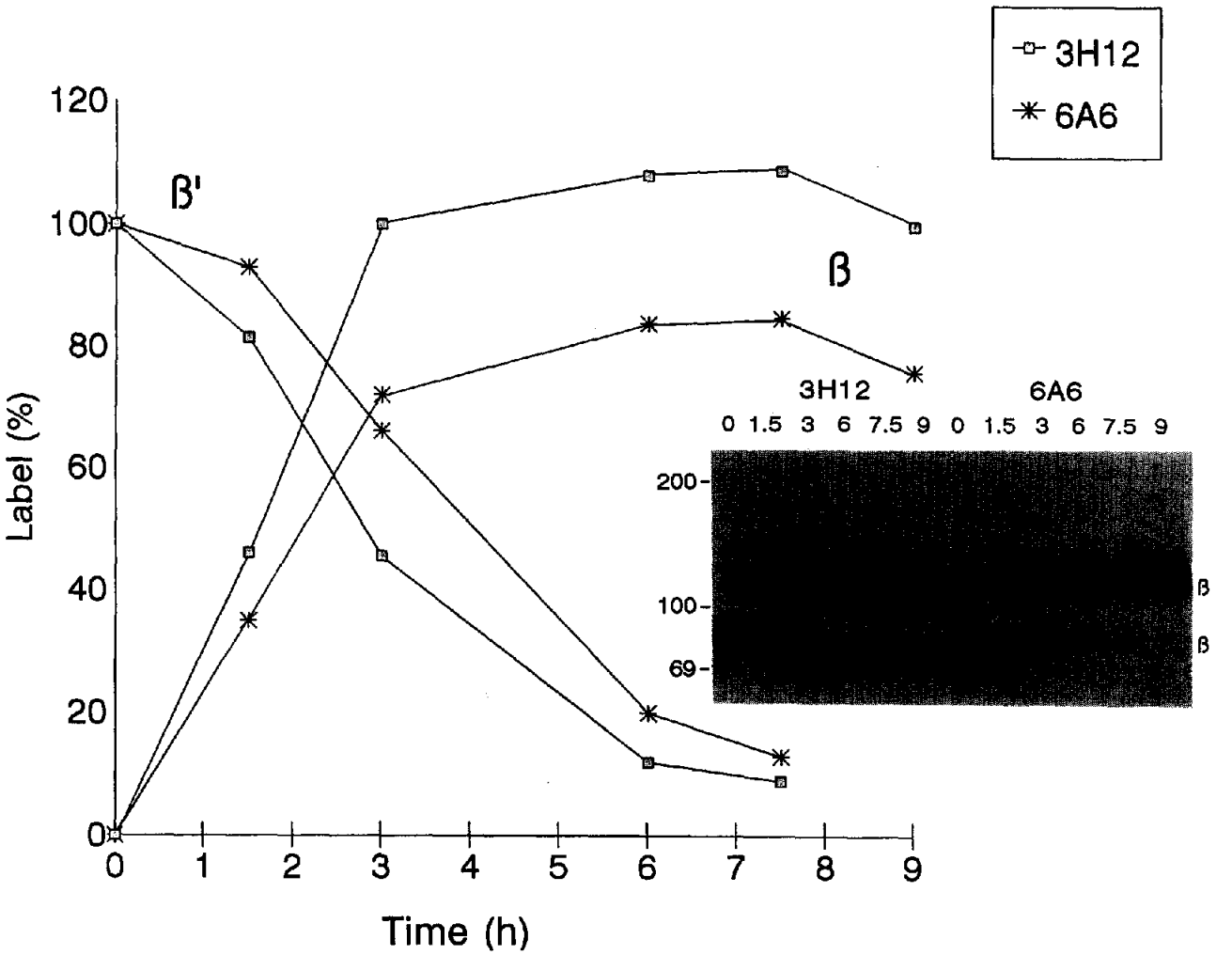

Fig. 4. Pulse–chase experiments of the β_1 integrin subunit in cell line 3H12 (α_{4m} expressing) and 6A6 (control). Double immunoprecipitates with Mab 7E2 (anti-hamster β_1) of stably transfected CHO-K1 cell lines 3H12 (α4m expressing) or 6A6 (control), metabolically labeled with [^{35}S]cysteine for 30 min and chased in unlabeled medium for the indicated time periods, were obtained as described in section 2. After endoglycosidase H digestion, samples were applied on a 6–10% SDS-PAGE. Dried gels were exposed to preflashed films to ascertain linearity of the obtained signals and autoradiographies were densitometrically scanned with a laser densitometer. The amount of mature β_1 at each time point is expressed as the percentage of the amount β_1 precursor present at time zero. Inset: autoradiography of the quantitated experiment. β', β_1-precursor; β, mature β_1-subunit.

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