Down regulation of talin alters cell adhesion and the processing of the $\alpha_5\beta_1$ integrin

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SUMMARY

The role of talin was addressed by down regulating its expression using an antisense RNA strategy. HeLa cells were transfected with a talin 5’ cDNA fragment under the control of the inducible human metallothionein promotor. Isolated clones displayed a decrease in talin level down to 10% of control. The reduction in talin expression dramatically slowed down the kinetics of cell spreading. Mock-transfected cells, spread out onto fibronectin, exhibited large peripheral adhesion plaques. In contrast, cells with reduced talin expression showed smaller focal contacts localized all over the ventral face, and displayed a marked decrease in the number of stress fibers. Immunoprecipitation experiments carried out with a polyclonal antibody on surface-labeled receptor indicated a shift in the mobility for both $\alpha_5$ and $\beta_1$ subunits. Surprisingly, $\beta_1$ integrin chains could not be detected by indirect immunofluorescence using monoclonal antibodies in talin deficient clones. Western blot analysis indicated the presence of two forms of $\beta_1$. We analyzed the processing of $\beta_1$ in normal and talin deficient cells using pulse chase experiments. Normal cells required a minimum of 5 hours for the processing of mature $\beta_1$, while the talin deficient AT22 clone showed that the $\beta_1$ precursor was slowly converted into a very low molecular mass product. Our data demonstrate that talin plays a central role in the establishment of cell-matrix contacts. In addition, down regulation of talin impairs the folding and processing of $\beta_1$ integrins.

Key words: antisense RNA, cell adhesion, focal adhesion, integrin, metallothionein promotor, talin, $\beta_1$ integrin processing

INTRODUCTION

Cell adhesion to extracellular matrix occurs through highly organized cellular structures named focal contacts, focal adhesions, or adhesion plaques (Woods and Couchman, 1988; Burridge et al., 1988; Beckerle and Yeh, 1990). Adhesion plaques are the transmembrane links formed via integrin receptors between the extracellular matrix and the cytoskeleton. The organization of this linkage has not yet been unravelled and is consequently poorly understood (Luna and Hitt, 1992). Some members of the integrin receptor family participate in the organization of focal contacts, whereas other members or other isoforms have never been found to be associated with these structures (Balzac et al., 1993). Furthermore heterogeneity in the composition of adhesion plaques has been suggested (Tranqui et al., 1993). On CHO cells grown onto fibronectin two types of contacts with distinct structural organizations and dynamics have been described: highly dynamic peripheral adhesion plaques which initiate stress fibers and more static thinner central contacts which are organized at an early stage in the adhesion process. The integrins are a large family of heterodimeric ($\alpha\beta$) transmembrane receptors (for review see Sastry and Horwitz, 1993). The C-terminal cytoplasmic domain of the $\beta$ chain interacts directly with specific cytosolic proteins such as talin and $\alpha$-actinin (Burridge et al., 1988; Otey et al., 1990). Conversely, the cytoplasmic C-terminal domain of the $\alpha$ subunit seems to control the ligand-dependent localization of integrin heterodimers into focal contacts (Briesewitz et al., 1993a). In addition to this structural role, recent data suggest that the clustering of integrins also plays an important role in outside-in signalling through the tyrosine kinase pp125 FAK (Schaller et al., 1992; Schaller and Parsons, 1993). Our increasing knowledge about the interactions of integrins with the extracellular matrix contrasts with the paucity of information on the proteins localized into focal contacts, their respective interactions, and their connections made with actin stress fibers. A number of proteins including talin, vinculin, tensin, pp125 FAK, zyxin and $\alpha$-actinin, are known to contribute to the overall organization of focal contacts (Burridge et al., 1988). Recent findings suggest a possible implication of focal contacts in outside-in and/or inside-out signalling. For example, some proteins such as paxillin, tensin and pp125 FAK become phosphorylated in response to cell adhesion to ligands of the extracellular matrix (Burridge et al., 1992; Bockholt and Burridge, 1993). The possible role of vinculin in the regulation of adhesion was recently addressed by the overexpression and suppression of vinculin in normal and transformed cells (Rodriguez Fernandez et al., 1992, 1993). Modulation of vinculin expression modifies the shape,
motility and adhesion-dependent growth ability of the cell, but has little effect on cell adhesion.

The ability of talin to interact with both cytoskeletal and membrane-associated proteins suggests that talin is involved in the establishment of transmembrane connections between the actin cytoskeleton and the extracellular environment. Such links occur at sites of cell-substratum adhesion. Thus, talin has a large protein with an apparent molecular mass of 235 kDa in mammals, composed of 2,541 amino acids, as deduced by Rees et al. (1990) from mouse cDNA sequence. Talin binds the cytoplasmic domain of the β1 and β3 integrin subunits and is associated with other cytoskeletal proteins such as vinculin (Horwitz et al., 1986; Sastry and Horwitz, 1993) and actin (Muguruma et al., 1990). Previous studies involving the microinjection of a monoclonal antibody into cells revealed that talin is required for the assembly of focal adhesions (Nuckolls et al., 1992). In addition to its obvious structural role, talin has a number of other intriguing features. It is a substrate of the Ca2+ activated protease calpain II found concentrated in focal contacts (Beckerle et al., 1987). Two proteolytic fragments of 47 kDa and 190–200 kDa are generated. Cleavage occurs at a specific site (Rees et al., 1990) for membrane associated talin whereas cytosolic talin remains intact (Tranqu and Block, 1995). The physiological significance of this cleavage remains unclear. Phosphorylation of the 47 kDa calpain N-terminal fragment of talin has been proposed as a possible signalling mechanism (Bertagnolli et al., 1993; Simons and Elias, 1993).

In order to study the effect of reduced talin expression on cell behavior, HeLa cells were transfected with an episomal inducible vector that controls the expression of a 5′ fragment of talin cDNA in the antisense orientation. This fragment was obtained from a λgt10 cDNA library screened with talin cDNA probe generated by RT/PCR on CHO cells. Two clones displaying a decrease in talin levels down to 10% of the controls were isolated. The phenotype of these clones suggested that talin is involved in the focal adhesion assembly and is required for the maturation and folding of the integrin with the β1 subunit.

MATERIALS AND METHODS

Materials

Monoclonal antibodies: anti-chicken talin (8d4), anti-human vinculin (hVIN1) and anti-actin against the C-terminal actin fragment were purchased from Sigma (St Louis, MO); the monoclonal anti-human β1 antibody K20 and the anti-α5 antibody (SAM-1) from Immunotech (Marseille, France). Polyclonal antibodies: anti-human β1 was obtained from Chemicon (Temecula, CA) and the anti-α5β1 from Gibco BRL (Gaithersburg, MD). For western blotting, the anti-human vinculin (V284) used was from Boehringer Mannheim (Germany). Oligonucleotides were synthesized by Appligene (Strasbourg, France) or Eurogentec (Seraing, Belgium). RNA was isolated according to the method of Gough (1988). DNA amplification was performed on a Dry Block PHC-3 (Techne, Inc). The partial talin cDNA was obtained from a CHO λgt10 cDNA library (Clontech, Palo Alto, CA). The cDNA fragments obtained by polymerase chain reaction and the λgt10 cDNA fragments were analyzed by double stranded plasmid DNA sequencing using the Sequenase kit (USB, Cleveland, OH).

Cell culture and DNA transfection

HeLa cells were from the Pasteur Institute, France. Cells were cultured in α-MEM supplemented with 10% heat inactivated fetal calf serum and 2 mM glutamine at 37°C in a 5% CO2 atmosphere.

For DNA transfection, 0.5×106 cells were seeded in 100 mm tissue culture dishes, grown overnight, and transfected with a total of 10 µg of plasmid using Transfectam (IBF, France). Adsorption and uptake of plasmid were carried out for 6 hours at 37°C. The day after transfection, the cells were split 1:10 or 1:50 and seeded. 24 hours later they were transferred into the selection medium containing 500 µg/ml of hygromycin (Sigma, St Louis, MO). After 10-20 days individual clones were isolated and expanded.

Plasmid construction

According to the sequence of mouse talin published by Rees et al. (1990) and based on the well-conserved amino acid sequences between mouse and hamster, two oligonucleotides were used as PCR primers to generate a fragment of talin cDNA surrounding an ATG region: primer Tal-1 5′CGTGAGCCGGATCCCAAGGCC3′ and primer Tal-2 5′TTCGCTCTCATACTCGGATC3′. Briefly, reverse transcription was carried out on total CHO RNA in 20 µl of buffer using 5 units of Moloney murine reverse transcriptase (Gibco BRL) and the primer Tal-2. The final volume during the amplification including the upstream primer Tal-1 was 100 µl after addition of all components. PCR thermal cycles were performed as follows: denaturation was performed at 94°C for 2 minutes, annealing at 50°C for 2 minutes, and polymerization at 70°C for 2 minutes. The reaction was initiated by adding 2.5 units of Taq DNA polymerase (Boehringer Mannheim Biochemicals), after which 40 thermal cycles were carried out as described above. The PCR reaction products were separated by agarose gel electrophoresis and the 192 bp DNA fragment was extracted by electroelution. The resulting DNA was blunt-ended, subcloned into the Smal site of pBluescript SK− (Stratagene, La Jolla, CA) for sequencing and used as a probe labeled with 32P for the ensuing screening of the CHO λgt10 cDNA library. The positive clones were purified by the plate lysate method (Maniatis et al., 1982). A cDNA fragment was isolated by EcoRI digestion. The resulting 0.9 kb fragment included 0.3 kb of the 5′ untranslated region and 0.6 kb of the coding region. A few differences between the nucleotide sequence of mouse and hamster talin were observed (manuscript in preparation). The cDNA fragment was purified by electroelution, blunt-ended and inserted either into the Smal-digested and dephosphorylated pBluescript SK− vector or into the PvuII-digested and dephosphorylated pMEP4 vector (Invitrogen Corp., Leek, Netherlands) in an antisense orientation. pMEP4 is an episomal vector conferring hygromycin resistance. Transcription of the cDNA is controlled by the inducible human metallothionein II gene enhancer/promoter while a termination signal is provided by the SV40 poly(A). The talin antisense expression vector was named pMEP4/AT2.

Episomal DNA preparation and Southern blotting

Episomal DNA was prepared by cell lysis with 0.6% SDS, 10 mM EDTA for 20 minutes. Then, 1 M NaCl was added and the mixture incubated for 5 hours. Two extractions with phenol/chloroform and ethanol precipitations were subsequently performed (Seed and Aruffo, 1987). One tenth of the preparation was used for PCR amplification with 2 oligonucleotide primers Tal-1 (bases matching the insert sequence) and oligonucleotide 667 5′CCGCGTGCACATCGTGCC3′ (bases matching the vector sequence). PCR amplification was carried out for 30 thermal cycles as previously described. PCR products were separated by electrophoresis on a 1.2% agarose gel.

Talin antisense RNA detection

Cytoplasmic RNA was digested with Promega RNase free DNase according to the manufacturer’s procedure in order to eliminate contaminating DNA. Messenger RNA (corresponding to 2 µg of total RNA) was isolated from the transfected cells by the method of Chomczynski and Sacchi (1987). The RNA was fractionated and transferred to nylon membranes as described (Maniatis et al., 1982). Hybridization and autoradiography were performed as described (Maniatis et al., 1982).
DNA probe used to detect the amplified product corresponded to the 
complementary strand: 5'-AAGCTGCCATGGTTGCCG3' and 5'-CTTGGAGTCATC-
CACCATG3'. Amplification steps were carried out using the 
PCR fragment generated from the plasmid pMPEP4AT2 with Tal1 and 
Tal2 (5'-AAGCTGCCATGGTTGCCG3' and 5'-CTTGGAGTCATC-
CACCATG3'). For pulse-chase labeling, the samples were split into two 30 
ml aliquots. To one aliquot, 5 ml (2 units) of endo F (Boehringer 
endoglycosidase F buffer. Then, the samples were split into two 30 
ml aliquots. To one aliquot, 5 ml (2 units) of endo F (Boehringer 
mannheim, diluted 1:5,000 in TBS/0.05% Tween-20/5% 
dried skimmed milk powder) for 1 hour and washed four times in 
TBS/0.05% Tween-20. Biotinylated proteins were visualized by 
chemiluminescence (ECL, Amersham).

Endoglycosidase F digestion
After cell-surface labeling with biotin, HeLa cells were lysed in RIPA 
buffer, and α5 and β1 polypeptides were immunoprecipitated. The 
immune complexes were washed three times in lysis buffer, rinsed 
once in endoglycosidase F buffer (50 mM KPO4, pH 7, 50 mM 
EDTA, 0.2% SDS, 0.5% Triton X-100) and resuspended in 60 
μl of endoglycosidase F buffer. Then, the samples were split into two 30 
ml aliquots. To one aliquot, 5 ml (2 units) of endo F (Boehringer 
mannheim) were added. To the other aliquot, 5 μl of buffer alone was added for mock digestion. The complexes were incubated at 37°C for 
22 hours. After digestion, SDS-PAGE sample buffer was added and the 
complexes were heated at 100°C for 5 minutes.

Indirect immunofluorescence
Cells were grown overnight on coverslips pre-coated with fibronectin, 
then rinsed three times with PBS, and fixed with 3% paraformalde-
yde in PBS containing 2% sucrose, for 10 minutes, at 37°C. When 
required, the cells were permeabilized at room temperature using 300 
μM sucrose, 50 mM NaCl, 3 mM MgCl2, 1% NP-40, 20 mM Hepes, 
pH 7.4, for 30 minutes (Dejana et al., 1988). After one wash with 
PBS, cells were incubated with primary antibodies in PBS containing 
1% BSA and 0.05% Tween-20 for 1 hour at 37°C. The cells were then 
washed four times with PBS/Tween-20 and incubated for another 
hour with rhodamine or FITC-conjugated goat anti-rabbit or anti-
mouse antisera in PBS containing 1% BSA and 0.05% Tween-20 at 
37°C. The coverslips were washed with PBS/Tween-20 and mounted onto glass slides in a Mowiol mounting solution. The slides were 
examined with a Nikon Microphot FXA microscope equipped for epi-
fluorescence.

FACS analysis
Cells were harvested with 1 mM EDTA, 0.1% Trypsin (w/v) and 
washed twice with PBS. The cells were fixed by 10 minutes incuba-
tion with 3% freshly prepared paraformaldehyde in PBS. The immune 
reaction was performed for 30 minutes at 4°C with the appropriate 
primary monoclonal antibody in Hanks’ balanced salt solution 
(HBSS) containing 0.3% saponin. It was followed by an incubation 
with the anti-IgG fluorescein-conjugated antibody (Jackson) in 
Hanks’ buffer containing 0.1% saponin. At each stage, the cells were 
washed three times in HBSS containing 0.1% saponin. Immunoflu-
orescence staining was analyzed on a FACScan flow cytometer
(Becton Dickinson) using a FACStar research software program (Lysis II). Background fluorescence was determined with a nonimmune mouse IgG control or a nonimmune rabbit antiserum. The data were displayed as cell number (ordinate) versus log fluorescence (abscissa). Quantification of fluorescence was done from acquisition in the linear mode and mean fluorescence intensity was expressed in FACS arbitrary values.

**Adhesion assays**

To determine cell adhesion on different substrates, nontreated 35 mm plates were coated with 10 μg/ml of vitronectin, laminin, or fibronectin for 2 hours at 37°C as previously described (Tranqui et al., 1992). Coating with collagen was done for 2 hours at 4°C to avoid its denaturation. Cell attachment (5x10⁵ cells per plate) took place at 37°C for 6 hours or 16 hours. Adherent cells were photographed under phase contrast and counted as flattened or round. The number of cells in a constant unit area (0.003 mm²) was estimated. All adhesion assays were run in triplicate. For each sample 2 areas were counted. Laminin, vitronectin and collagen are purchased from Sigma. Fibronectin was purified from bovine plasma according to the method of Engvall and Ruoslahti (1977).

**RESULTS**

**Production of HeLa cell lines transfected with talin antisense cDNA**

HeLa cells were transfected with the episomal vector pMEP4/AT2 designed to produce antisense talin RNA under control of the inducible human metallothionein II promotor (see Materials and Methods). The 0.9 kb λgt10 talin cDNA fragment inserted into the pMEP4 vector included 0.3 kb of the 5’ untranslated region, the ATG start codon and the 0.6 kb of the translated sequence (Fig. 1). After 10 days of transfection, 50 hygromycin-resistant colonies were selected. Numerous HeLa clones appeared to be hygromycin-resistant at first glance but turned out to die after the second screening: they did not resist long-term culture with antibiotic. Among the resistant clones, two of them (designated HeLa/AT21 and HeLa/AT22, respectively), contained the episomal pMEP4/AT2 plasmid and were further characterized. Identification of the vector was performed using the polymerase chain reaction (PCR). The results are shown in Fig. 2. Briefly, amplification was carried out using the vector extracted from the transfected HeLa cells as a matrix, and the primers 667 and

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**Fig. 1.** Cloning strategy. The primary structure of talin deduced from the complete sequence of the mouse protein (Rees et al., 1990) is shown and the site of cleavage by calpain II into two proteolytic fragments (between residues 433-434) is indicated. Black boxes represent the two putative vinculin binding sites (Gilmore et al., 1992). The positions of some of the unique restriction sites are indicated with vertical lines above the cDNA of talin. According to the published sequence, two oligonucleotides (TAL-1 and TAL-2) were designated to generate the probe by RT/PCR (dotted box) which was used for the screening of ATG-containing talin cDNA fragments. The talin fragment isolated from the λgt10 library contains 0.3 kb of the 5’ untranslated sequence followed by 0.6 kb of the 5’ translated sequence. The whole of this fragment was cloned into the expression vector pMEP4 in the antisense orientation.

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**Fig. 2.** PCR screening of the transfected clones. (A) PCR reactions were performed on a DNA preparation designed to extract any episomal vector from HeLa cells. From the left, lane 1 is a negative control using nontransfected HeLa cells. In lane 2 HeLa cells transfected with pMEP4/AT2 are used (clone HeLa/AT22). A PCR product in the QUIAGEN-purified expression vector pMEP4/AT2 constitutes the positive control (lane 3). It generates the expected fragment of 550 bp. 20 μl aliquots of 50 μl reactions are run on a 1.2% agarose gel. Lane 4 contains the molecular size markers (1 kb ladder, BRL). (B) The oligonucleotides TAL-1 and 667 are localized on talin cDNA and parental pMEP4 expression vector, respectively. The black box represents the human metallothionein II promotor which controls the transcription of talin fragment in the antisense orientation (gray box).
Talin-1 matching the MTII promotor sequence and the insert sequence, respectively (Fig. 2B). Therefore, amplification of DNA could not occur from the endogenous talin gene which reveals that the λgt10 talin cDNA fragment was truly inserted into the antisense position. This PCR generated the expected fragment of 550 bp (Fig. 2A) identical to the PCR-fragment produced from a control reaction using the original expression vector pMEP4/AT2. Conversely, under the same experimental conditions, we were unable to generate this fragment either with wild-type HeLa cells or with pMEP4-transfected HeLa cells.

Expression of the talin antisense RNA was determined by a RT/PCR reaction (Fig. 3) using an oligonucleotide complementary to the expected antisense RNA for the first strand synthesis. The generated 329 bp fragment was detected after hybridization with a talin specific probe (189 bp) synthesized by PCR. This 329 bp fragment is localized between nt -11 and nt 318 whereas the probe is from nt 96 to nt 285 (relative to the translation initiation). This 329 bp fragment was present in the AT22 clone but not in wild-type HeLa cells or when hybridized to products generated by PCR without reverse transcriptase. GAPDH mRNA amplification was used as an internal control in each reaction (data not shown).

Down regulation of talin expression in transfected cells

Western blot experiments and flow cytometry analysis of HeLa/AT22 or AT21 cells disclosed an important decrease in talin expression compared to wild-type HeLa cells or pMEP4-transfected HeLa cells (mock transfected). Flow cytometry analysis was carried out with fixed and saponin-permeabilized cells after an incubation with the monoclonal anti-talin primary antibody 8d4 (Fab’2). An important decrease in the fluorescence of HeLa/AT22 cells as compared to pMEP4 transfected cells was observed (Fig. 4A). Overnight addition of cadmium chloride to the culture medium resulted in a further decrease in the fluorescence of HeLa/AT22 cells (Fig. 4B) but there was no effect on pMEP4 transfected HeLa cells, i.e. mock-transfected cells (not shown). Quantification of the FACS results for HeLa/AT22 indicated a decrease, compared to mock transfected cells, of 90% and 80% in the talin related fluorescence with or without cadmium induction, respectively (Table 1). On western blot experiments performed with 50 μg of proteins from whole-cell extracts (Fig. 4C and Table 1) and 8d4 anti-talin antibody, AT22 cells displayed a decrease in talin synthesis whereas levels of other cytoskeletal proteins such as vinculin and actin were not altered. After the normalization of the data using actin expression, the densitometry of the stained band corresponding to talin indicated a decrease in talin content of about 60% and 38% compared to talin levels in mock transfected cells, with or without cadmium induction, respectively (Table 1). Similar figures were obtained with...
HeLa/AT21 cells (not shown). After metabolic labeling and talin immunoprecipitation, quantification given by a phosphorimager showed a decrease in talin content of 80% and 54% compared to talin levels in mock transfected cells, with or without cadmium, respectively (Fig. 5 and Table 1). Indeed, these data indicated that our antisense talin construct efficiently and specifically downregulated talin expression in HeLa cells. Although basal level transcription of the human metallothionein II promoter was relatively high and gave an already significant decrease in talin expression without any induction, the addition of cadmium to the culture medium further decreased the intracellular talin level.

**Phenotype and adhesive properties of HeLa/AT clones**

As the presence of our antisense DNA construct did perturb the expression of talin in HeLa cells, we evaluated their degree of spreading on different substrates. Untreated 35 mm dishes were coated with collagen, laminin, vitronectin and fibronectin at a concentration of 10 μg/ml in PBS as described in Materials and Methods. Although the morphologies of antisense expressing cells and wild-type cells varied widely, the cells were simply sorted into two categories: either round or spread-out. The number of cells of each type was counted at 1 hour, 6 hours and 16 hours after the beginning of adhesion at 37°C. A cell was scored as spread-out if it displayed a thin lamella while cells lacking these structures were scored as round. Usually the latter group of cells were surrounded by a phase-light region or refringent region. Spread cells could be maintained for 3 days at 37°C without subsequent morphological modifications.

Cell spreading kinetics differ depending on the substrate and were fastest on fibronectin-coated dishes. After 6 hours, 65% of spread cells were counted on fibronectin whereas on laminin, vitronectin and collagen they reached only 40%, 10%, and 15%, respectively (Fig. 6A,B). Under identical experimental conditions, wild-type or mock transfected HeLa cells achieved almost complete spreading on fibronectin within 15 minutes. Phase contrast microscopy at high magnification revealed that the cells of antisense transfected clones (with a reduced talin level) were poorly spread onto the substrates. Conversely, mock-transfected cells expressing normal talin levels spread normally and had a typical morphology indistinguishable from that of the wild-type cells. After 48 hours at 37°C, full spreading of HeLa/AT cells was almost achieved on all the substrates tested but collagen. With this latter substrate, 85% of the cells kept a round morphology (Fig. 6A). However, a targeted reduction in talin expression was sufficient for conferring changes in cell shape even on fibronectin. The transfectected cells were attached but poorly spread on the substrate (Fig. 6A). Addition of cadmium at non-lethal concentration induced the rounding up of HeLa/AT cells seeded onto tissue culture treated plastic within 16 hours, but has no effect on wild-type HeLa or mock transfected HeLa under our experimental conditions (Fig. 7).

**Analysis of focal adhesions and cytoskeletal organization in HeLa/AT cells**

It is noteworthy that most cells displaying a round morphology were lost from the coverslip during the process of fixation, permeabilization and immunostaining. Consequently, immunofluorescence microscopy revealed only cells displaying a spread-out morphology. Furthermore, when round cells were still present on the coverslip at the end of the immunostaining process, we were unable to detect any organization of focal adhesions. Thus the immunostaining experiments were performed with cells spread on fibronectin for 48 hours without cadmium. Under these experimental conditions, we knew that the basal level of the metallothionein promoter was high enough to induce both a reduction in spreading capacity and down regulation of talin expression. In mock-transfected HeLa cells, talin and vinculin were found in focal adhesion localized mainly at the cell margins (Fig. 8A,C). Identical patterns were observed with wild-type cells (not shown). On the other hand, immunostaining of talin and vinculin revealed that the organization of focal adhesions was strongly modified in HeLa/AT cells (Fig. 8B,D). Double staining of AT22 cells showed a colocalization of talin and vinculin (Fig. 9) indicating that these small adhesion plaques still constituted both proteins. While mock-transfected HeLa cells had large peripheral adhesion plaques incorporating β1 integrin chains (Fig. 8E), the HeLa/AT cells exhibited a large number of smaller focal adhesions localized all over the ventral face. Surprisingly, the HeLa/AT clones gave no immunostaining with the K20 anti-β1 monoclonal antibody (Fig. 8F). The staining of actin microfilaments by fluorescein-conjugated phalloidin showed a cortical actin network and a much reduced number of stress fibers.

![Fig. 5. Immunoprecipitation of talin after metabolic labeling. Following metabolic labeling with [35S]methionine and [35S]cysteine (50 μCi/ml) for 3 hours, the cells were lysed with RIPA buffer. Talin was subsequently immunoprecipitated with the monoclonal antibody 8d4 from equal amounts of proteins of total cell lysates determined by micro BCA assay. Lane 1, talin from control cells; lane 2, talin from induced AT22 cells; lane 3, talin from non induced AT22 cells.](image-url)
fibers at the periphery of the talin deficient cells (Fig. 8H) compared to control cells (Fig. 8G).

**The β1 integrin subunit is present at the surface of talin deficient cells in a distinct conformational state**

The lack of β1 detection observed by immunostaining (Fig. 8F) was confirmed by FACS analysis using the same monoclonal antibody (K20) directed against the extracellular domain of the β1 subunit (Fig. 10). Another anti-β1 monoclonal antibody (clone P4C10-Gibco-BRL) was assayed and gave an identical result (data not shown). Immunoprecipitation experiments were carried out with a polyclonal antibody on surface labeled receptor using both wild-type and AT22 cells. On wild-type HeLa cells, α5 and α1 subunits coimmunoprecipitated with the β1 chain. Conversely, in the AT22 clone, both α5 and β1 subunits underwent processing events that resulted in shifts to lower relative molecular mass (Fig. 11). These abnormal chains were named α5' and β1', respectively. There was no major change in the total amounts of fibronectin receptor subunits expressed and inserted into the plasma membrane. Altogether these data indicated that β1' in association with α5' was still present at the surface of talin deficient cells. However, the β1' as well as α5' subunit appeared to be modified since there was loss of epitopes recognized by several monoclonal antibodies and a mobility shift on SDS-PAGE. We tried to detect the presence of the α5' subunit at the cell surface of the HeLa/AT22 cells spread-out onto fibronectin (Fig. 12). A monoclonal antibody raised against α5 (clone SAM1) revealed that this subunit was localized into focal adhesions (Fig. 12A). The same levels of α5 subunit were found by FACS analysis on HeLa/AT, or mock transfected cells (Fig. 12B,C).

Although Fig. 11 shows that the α1 expression level was dramatically reduced, immunoprecipitation of surface labeled proteins with anti-α1 antibodies shows the still remaining α1 subunit associated with the β1 chain. But in the same way α1/β1 integrin shows a shift in the mobility on SDS-PAGE (not shown).
Talin down regulation alters the processing of β1 integrins

Western blot experiments were performed on cell lysates from mock, HeLa/AT21 or HeLa/AT22 cells, using a specific (affinity purified) polyclonal antibody raised against the cytosolic domain of β1 (Chemicon). Fig. 13 shows that, despite the lack of its immunofluorescence detection, the β1 chain was still expressed by all these cells as described by immunoprecipitation experiments. Under non reducing conditions a single band corresponding to the apparent molecular mass of 130 kDa of β1 was detected in mock cell lysates with or without cadmium added. Conversely, without any cadmium added, two
bands were detected by the anti-β1 antibodies in both talin deficient HeLa/AT21 and AT22 clones (Fig. 13). The lower molecular mass chains might correspond to some forms of pro-β1 chains (Heino et al., 1989). Addition of cadmium for 24 hours to HeLa/AT cells resulted in a dramatic increase in the lower band and the complete loss of the higher chain (Fig. 13). A cell lysate of mock-transfected cells from a 24 hour culture in suspension was also analyzed (Fig. 13). A single 130 kDa chain was detected in this lysate. This indicated that the putative pro-β1 accumulation was not due to a defect in cell adherence. In all the cells tested with or without cadmium induction, no significant changes in β1 RNA levels were found as compared to GAPDH mRNA levels taken as internal controls (not shown). Thus, a decrease in talin expression did not affect expression of the β1 gene. The hypothesis that the β1 integrin was not processed correctly in talin deficient cells was investigated with non-induced cells by pulse-chase experiments (Fig. 14). The pulse-chase experiments followed by the immunoprecipitation of the β1 chain with an anti-C-terminal domain (Chemicon) indicated that normal cells required a minimum of 5 hours to allow the conversion of pro-β1 (100 kDa) into mature β1 (130 kDa). Conversely, AT22 cells showed a progression of a β1 precursor form with an apparent molecular mass of approximately 100-110 kDa into a new form of β1 with very low molecular mass of about 90 kDa. Presumably, this end product corresponded to the accumulated β1 form detected by western blot analysis upon cadmium addition. As indicated in pulse chase experiments, AT22 cells showed the conversion of proβ1 into β1 but never the maturation of the proβ1 into β1. Consequently, the protein doublet detected in AT cells by western blot (without cadmium induction) might correspond to the proβ1 and the β1.

Pulse chase experiments also indicated that in wild-type HeLa cells proα5 and proα1 coimmunoprecipitated with proβ1 and were converted into α5 and α1 subunits, respectively, in about 3 hours. For AT22 cells, a lower molecular mass α band coimmunoprecipitated with proβ1 and was identified as a modified pro α5 subunit (Fig. 14). This chain was quickly processed (1 hour) into abnormal lower molecular mass α5.

**Decrease in talin results in partial glycosylation of fibronectin receptor**

To determine whether the modification of integrin processing was related to a defect in glycosylation, immunoprecipitates of cell-surface-biotinylated α5 and β1 were treated with endoglycosidase F and analyzed by SDS-PAGE under non-reducing conditions. Endoglycosidase F, which cleaves both the high-mannose and complex forms of the N-linked oligosaccharides from the polypeptide backbone, induced a shift in the mobility of cell surface α5 and β1. In wild-type...
HeLa cells, the apparent molecular masses of \( \alpha_5 \) and \( \beta_1 \) subunits drop from 155 kDa to 120 kDa and from 130 kDa to 90 kDa, respectively (Fig. 15, lanes 1, 2 and 3, 4), whereas in AT22 cells, the apparent molecular mass of \( \alpha_5 \) was reduced from 135 kDa to 120 kDa (Fig. 15, lanes 5, 6) and for \( \beta_1 \), from 100 kDa to 90 and 80 kDa. Thus, treatment with endoglycosidase F yielded the same end product of 120 kDa for \( \alpha_5 \) indicating that the defect in the processing of this subunit observed in AT2 clones was an incomplete N-glycosylation. On the other hand, endo F digestion of \( \beta_1 \) produced a 90 kDa band found in both wild-type HeLa cells and AT22 clone and a 80 kDa band found solely in AT22. It is likely that the 90 kDa represents the unglycosylated \( \beta_1 \). Consequently, the 80 kDa band that accumulated in AT22 cells is a truncated \( \beta_1 \). For this latter subunit, the defect in the processing results from both partial glycosylation and proteolytic cleavage.

**DISCUSSION**

An experimental approach using transfected HeLa cells with a 0.9 kb antisense talin cDNA (including a part of the 5’ untranslated sequence) was designed to analyze the function of talin in cell adhesion and regulation. Since inhibition of expression of the proteins involved in cell adherence might be lethal, we chose the expression vector pMEP4. Upon cadmium addition, the human metallothionein II promotor drives the transcription of the antisense RNA from the inserted antisense talin cDNA. The origin of pMEP4 replication allowed a great number of episomal copies of the plasmid in HeLa cells. Therefore, high levels of antisense RNA required for the inhibition of expression of the targeted protein were produced. Indeed, when the construct was used to transfet CHO cells in which only replication of genome-integrated plasmid occurred, a very transient modification of cell adhesion was observed (personal unpublished data). After the transfection of HeLa cells, two independent clones containing the episomal vector were isolated and fully characterized. The clones exhibited identical phenotypes and were named HeLa/AT21 and AT22. Western blots as well as cytofluorimetric analysis and immunoprecipitation after metabolic labeling indicated that the level of talin could be reduced down to 10-40% of that normally found in wild-type or mock-transfected cells. This decrease was cadmium dependent, which indicates that down regulation was due to antisense RNA synthesis under the control of the hMT II promotor.

Our present results suggest that talin deficiency causes a defect in cell adherence. These cells not only spread poorly onto various substrates, but also show a dramatic decrease in their speed of adhesion and spreading (Fig. 6). Indeed, it took at least 48 hours at 37°C for these cells to be fully spread onto fibronectin (the most efficient substrate promoting adhesion of HeLa/AT cells), while only 15 minutes were required for wild-type HeLa cells and mock-transfected cells under identical conditions. After 48 hours at 37°C, the typical pattern of focal adhesion was markedly different in HeLa/AT clones compared to mock-transfected or wild-type cells (Fig. 8). Whereas normal cells exhibited a small number of large peripheral focal adhesions, HeLa/AT cells exhibited a large number of smaller
focal adhesions localized all over the ventral face. Adhesive structures in clones with decreased talin levels appeared to have an apparent normal structure as seen by immunofluorescence using antibodies against talin and vinculin. These patterns of large peripheral or small focal adhesions have been previously described as late and early stages of CHO cell adhesion, respectively (Tranqui et al., 1993). They were also observed in HeLa cells (not shown). Since the kinetics of adhesion were so slow with HeLa/AT cells, it is likely that the distribution of focal adhesions in these clones reflected solely an early stage of cell spreading rather than an abnormal organization. We suggest that, in talin deficient cells, the final state of spreading with large peripheral adhesion plaques could never be reached. With HeLa/AT cells, a straightforward interpretation of the slow kinetics of adhesion can be proposed: the recruitment of talin into focal adhesions may become a stringent limiting step because of the low amount of this protein in the cytosol. The role of talin in the establishment of focal adhesions suggested by our results is consistent with microinjection experiments using the anti-talin monoclonal antibody 8d4 (Nuckolls et al., 1992). Finally, a further decrease in talin expression by addition of cadmium induced the rounding up of AT21 and AT22 clones, whereas wild-type HeLa or mock transfected cells remained spread under identical experimental conditions. Similarly, down regulation of vinculin expression by antisense transfection confers a moderate defect in cell adhesion with smaller vinculin-positive plaques (Rodriguez-Fernandez et al., 1993). The organization of the cytoskeleton and focal contacts was also modified in HeLa/AT cells. Although these cells exhibited focal adhesions, fewer and shorter stress fibers remained, as though a high talin concentration were required for the nucleation of new stress fibers.

Surprisingly, the adhesion deficiency of HeLa/AT clones varied according to the extracellular matrix protein used (Fig. 6). These differences suggested that modifications of the integrin receptors might also occur upon down regulation of talin expression. Indeed, a loss of epitopes detected by immunofluorescence studies indicated that the β1 chain was in a distinct conformational state named β'1. The loss of detection was not due to a down regulation of β'1 expression, since PCR did not reveal any significant changes in β'1 mRNA levels and western blots showed that the chains were synthesized within the cells (Fig. 13). However, distinct β'1 forms were found in AT clones with lower apparent molecular masses that might correspond to precursors of β1 (Heino et al., 1989; Hotchin and Watt, 1992). Abnormal processing of β'1 integrins was confirmed by pulse chase experiments (Fig. 14). In wild-type HeLa cells normal processing of β1 took at least 5 hours. Conversely, in the AT22 clone, at time zero the precursor of β'1 was detected with a slightly higher molecular mass. This form was slowly converted into a low molecular mass form of the β1 chain. The defect in β'1 processing is probably a consequence of the genetic down regulation of talin since it was observed with the two independent clones HeLa/AT21 and HeLa/AT22 but not with mock-transfected HeLa cells. In the latter cells normal β1 chains and no pro-β1 were immunodetected in western blots. Furthermore, when the expression of antisense RNA was induced by a non lethal concentration of cadmium, the abnormal lower molecular mass β'1 form accumulated in HeLa/AT. Our results suggest that a more...
dramatic decrease in talin level resulted in the amplification of b1 processing defects. An alternative explanation for the modifications of the b1 chain could be the lack of adhesion with sufficient mechanical strength. Indeed, substrate adhesion has been reported to modulate integrin gene expression and antigenic sites of the receptors (Frelinger et al., 1988; Chen et al., 1992). However, the observation that after 24 hours of culture in suspension, mock-transfected cells presented b1 chains indistinguishable from those of adherent mock-transfected cells, ruled out this hypothesis.

Pulse chase experiments indicated that α1, α5 and β1 subunits underwent processing events that resulted in shifts to a higher relative molecular mass, this suggesting that post-translational modifications were occurring. According to the treatment with endo-F, these modifications were mostly due to glycosylation. The same treatment performed on AT22 cells showed that the mobility shift for b1 and α5 subunits compared to wild-type HeLa cells was due to partial glycosylation and also to proteolytic cleavage concerning the b1 chain. Furthermore, the monoclonal antibody K20 has been reported to recognize an epitope localized in the region 426-587 of the b1 chain (Takada and Puzon, 1993). This epitope is no longer recognized in the case of b1 integrin synthesized in AT22 cells, which is possibly related to the loss of glycosylation as 3 sites of potential N-linked glycosylation are localized in this region (Argraves et al., 1987). This would mean that the epitope bound by monoclonal antibody K20 developed relatively late during biosynthesis and needs post translational modification such as N-linked glycosylation in order to be recognized. Although α5b1 (and even α1b1; data not shown) synthesized in AT clones was abnormally processed, the low molecular mass final product reached the cell surface since it was detected after immunoprecipitation of the surface labeled proteins by anti-α5b1. Intermediate forms of the fibronectin receptor subunits were found on the surface of AT22 cells, hence complete processing of receptors into mature forms was not necessary for insertion into the plasma membrane. However, it must be pointed out that glycosylation might influence the receptor affinity for ligand on VLA integrins. As reported here, the molecular changes in the b1 subunit in HeLa/AT clones had only a limited influence on the final state of cell spreading onto laminin and fibronectin. Conversely, under identical experimental conditions, HeLa/AT spreading never occurred onto collagen which can be explained by the down regulation of the α1 subunit (Fig. 11 and Fig. 14). The b1 integrin subunit is associated with various α chains to form receptors for fibronectin (α4b1 and α5b1), laminin (α1b1, α2b1, α3b1 and α6b1), collagen (α1b1, α2b1, α3b1, and α4b1) and vitronectin (αvB1) (Hemler, 1990; Hynes, 1992). The coexistence of multiple α subunits capable of assembly with a common β1 subunit to form a repertoire of integrin complexes with distinct adhesive specificities, and the fashion in which these complexes are assembled might be controlled by the glycosylation.

According to our data, down-regulation of talin seems to correlate with a defect in b1 integrin processing. This raises the possibility that talin could act as a chaperon, helping the folding of integrin receptors that will be normally associated with it. This is apparently not consistent with the fact that some integrin heterodimers with deletions in the transmembrane and cytoplasmic domains have been successfully expressed into host cells secreted in a functional soluble form (Briesewitz et al., 1993b). However, the total amount of the truncated integrin remains still very low compared to the level of expression of the normal protein (Frachet et al., 1992). Finally, biosynthetic studies using stable-expressing cell lines demonstrate that the soluble and the native heterodimers are formed independently. These studies strongly suggest that the cytoplasmic domains of α and/or β subunits are involved in the assembly of native heterodimers (Briesewitz et al., 1993b). Our results add another piece of evidence in support of a role for talin in integrin assembly.

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REFERENCES


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