FEBS Letters 484 (2000) 194–198 FEBS 24261

Anomalous dystroglycan in carcinoma cell lines

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Received 9 August 2000; revised 21 September 2000; accepted 4 October 2000

First published online 24 October 2000

Edited by Felix Wieland

Abstract Dystroglycan is a receptor responsible for crucial interactions between extracellular matrix and cytoplasmic space. We provide the first evidence that dystroglycan is truncated. In HC11 normal murine and the 184B5 non-tumorigenic mammary human cell lines, the expected β-dystroglycan 43 kDa band was found but human breast T47D, BT549, MCF7, colon HT29, HCT116, SW620, prostate DU145 and cervical HeLa cancer cells expressed an anomalous ≈31 kDa β-dystroglycan band. α-Dystroglycan was udetectable in most of the cell lines in which β-dystroglycan was found as a ≈31 kDa species. An anomalous ≈31 kDa β-dystroglycan band was also observed in *N*-methyl-*N*-nitrosurea-induced primary rat mammary tumours. Reverse transcriptase polymerase chain reaction experiments confirmed the absence of alternative splicing events and/or expression of eventual dystroglycan isoforms. Using protein extraction procedures at low- and high-ionic strength, we demonstrated that both the 43 kDa and ≈31 kDa β-dystroglycan bands harbour their transmembrane segment.

Key words: Dystroglycan; Protein processing; Carcinoma; Immunodetection; Reverse transcriptase polymerase chain reaction

1. Introduction

Dystroglycan is a transmembrane protein that binds extracellular matrix proteins, proteoglycans and cytosolic proteins, and is formed by two subunits, α-dystroglycan (extracellular) and β-dystroglycan (transmembrane). The two subunits, which form a tight non-covalent complex, are encoded by the same gene. Dystroglycan is expressed in a wide variety of tissues and has important functions during early embryogenesis, for the mechanical stabilisation of muscles and also for bacterial and viral infections [1,2]. The highly and heterogeneously glycosylated cell surface-associated α-dystroglycan has a size which ranges from 120 to 180 kDa, depending on different amounts of glycosylation [3,4]. It was recently demonstrated that the glycosylation pattern of α-dystroglycan changes during embryogenesis [5]. On the contrary, β-dystroglycan was identified as a 43 kDa band, sometimes accompanied by a ≈31 kDa band, which presumably originates from proteolytic fragmentation and/or alterations in its glycosylation pattern [6–9]. α-Dystroglycan binds with high affinity to extracellular matrix proteins and proteoglycans as laminins, agrin and perlecan [10–14], whereas the cytoplasmic tail of β-dystroglycan is involved in several interactions with a number of proteins (dystrophin and its isoforms, Grb2, rapsyn) [15–17]. Dystroglycan has the role of connecting the extracellular matrix network to the cytoskeleton [18] and is possibly involved in the regulation of signalling pathways [19].

In the past years, several studies have analysed the role of cell–matrix interactions at the onset and/or during development of cancer [20,21]. In particular, human mammary gland carcinoma cell lines represent a paradigmatic model system for such analysis [22].

In the present study, we have investigated the expression pattern of dystroglycan in a panel of both normal and cancer cell lines. Our results indicate that dystroglycan is diminished in size in malignant cell lines due to post-translational processing events.

2. Materials and methods

2.1. Cell cultures and animals

The HC11 mouse mammary epithelial cell line was clonally derived from a spontaneously immortalised mammary epithelial cell culture originally established from a midterm pregnant BALB/c mouse [23] and was grown and maintained in RPMI 1640 medium (RPMI; Gibco, Merelbette, Belgium) supplemented with 10% heat-inactivated foetal bovine serum (Gibco). The immortalised non-tumorigenic human mammary epithelial cell line 184B5 (a gift from M. Stampfer, Lawrence Berkeley Laboratory, Berkeley, CA, USA) was grown in supplemented MCDB 170 medium (Clonetics Corp., San Diego, CA, USA) as described elsewhere [24]. All the other cell lines used for this study were obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured as recommended by the supplier.

The source and housing of the animals used in this study have been described previously [25]. Briefly, mammary cancers were induced in 50-day-old virgin-female Sprague-Dawley rats with a single intravenous injection of 50 mg *N*-methyl-*N*-nitrosurea (NMU) per kg body weight via the jugular vein, as described [25]. A total of seven tumours were analysed. They were all malignant tumours with a predominant cystic papillary adenocarcinoma pattern.

2.2. Total protein extract

Two different lysis buffers were used: (1) TX-100: 1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 0.5% NP-40, 1 mM benzamidine, 25 μg/ml leupeptin; (2) 20 mM Tris–HCl, pH 7.5, 2 mM EGTA, 2 mM EDTA, 6 mM β-mercaptoethanol supplemented with protease inhibitors, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40. Exponentially growing cultures of the indicated cell line were collected and cell pellets were resuspended in the lysis buffer. The incubation was performed on

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0014-5793/00/$20.00
PII: S0014-5793(00)02157-8

ice with an occasional agitation. The cell lysates were centrifuged for 15 min. The supernatant corresponds to the total cell lysate whose concentration was determined by BCA protein assay (Pierce).

A rabbit cardiac muscle membrane extract, partially purified in non-dissociating conditions using succinyl-wheat germ agglutinin (sWGA) agarose (Vector), was used as a positive control for β-dystroglycan. Frozen rabbit heart was homogenised with Physcotron NS-10 in the buffer A containing 0.5 M NaCl, 50 mM Tris–HCl pH 7.4, 1.5 mM benzamidine, 0.25 mM PMSF, 2.5 μg/ml leupeptin, 2.5 μg/ml pepstatin A, 2.5 μg/ml aprotinin, 1% digitonin. After centrifugation at $100\,000\times g$ for 30 min at 4°C, the supernatant was incubated overnight at 4°C with a 50% batch of sWGA agarose beads. The beads were washed extensively with buffer B which had the same composition as buffer A, except for a reduced concentration of digitonin (0.1%), and then eluted with 0.35 M *N*-acetyl-glucosamine.

For low-ionic strength extractions, a single cell pellet was incubated and mechanically lysed in 20 mM HEPES/NaOH pH 7.4, 10 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 10 μg/μl leupeptin, 1 mM PMSF, 1 mM NEM, 1 mM benzamidine. The lysate mixture was separated in two different tubes and centrifuged ($14\,000\times g\times 10$ min, 4°C) and the supernatant was collected. One pellet was resuspended in TX-100 lysis buffer for Western blot analysis, whereas the other was resuspended in high-ionic strength conditions (1 M KCl, 5 mM phosphate-buffered saline (PBS), 1 mM EDTA, 10 μg/μl leupeptin, 1 mM PMSF, 1 mM NEM, 1 mM benzamidine, pH 7.5–8). After centrifugation, a new supernatant fraction was collected and dialysed in 10 mm Tris–HCl pH 7.5. The pellet fraction was washed using 20 mM HEPES/NaOH pH 7.4 and then resuspended in TX-100 lysis buffer. Supernatant and pellet fractions were analysed by SDS–PAGE and Western blot.

2.3. Gel electrophoresis and Western blot

Mouse monoclonal antibodies NCL-43DAG (clone 8D5) raised against the last C-terminal 15 amino acidic residues of the β-dystroglycan cytoplasmic domain from Novocastra (Newcastle-upon Tyne, UK) were used to detect β-dystroglycan, whereas for detection of α-dystroglycan a mouse monoclonal VIA4-1 was used (Upstate Biotechnology, USA). Proteins present in the lysates were boiled for 3 min at 90° C in sample buffer and subjected to SDS–PAGE electrophoresis on 4–15% gels. For Western blot analysis proteins were electrophoretically transferred to a nitrocellulose membrane at 35 V overnight. The nitrocellulose was blocked with 3% milk powder in Tris-buffered saline (TBS; 150 mM NaCl, 10 mM Tris–HCl buffer pH 7.4) for 2 h and incubated for 1 h with the monoclonal antibody in use, diluted 1:25 in TBS containing 3% bovine serum albumin. After washing in TBS containing 3% milk powder and 0.1% Tween 20, the blots were incubated for 30 min with anti-mouse antibodies horseradish peroxidase-conjugated (Pierce) diluted 1:5000 in the same buffer. After one wash for 20 min in 3% milk powder T-PBS and several washes in T-PBS without milk, the filters were incubated with the detection solution of the luminol-based ECL system (Pierce). Fluorographies were carried out at room temperature by exposing nitrocellulose sheets to Kodak films.

2.4. Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was isolated from pelleted cells using the Rneasy Mini Kit (Qiagen), obtaining a yield of 30–40 μg from $0.5–1\times 10^6$ cells. The concentration and purity of RNA was determined spectrophotometrically measuring the A^{260nm}/A^{280nm} ratio. For cDNA synthesis, the Enhanced Avian RT-PCR kit (Sigma) was used. Usually, ≈5 μg of total RNA was incubated in DEPC-treated water and 2.5 μM oligo-dT, and preheated at 70°C for 10 min, then the mixture was incubated in the presence of Avian reverse transcriptase at 42°C for 1 h following the conditions recommended by the manufacturer. PCR reactions contained 5 μl from the cDNA reactions, 200 μM dNTPs, ≈0.4 μM of each primer, 1×Red-Taq polymerase buffer and 2.5 U of Red-Taq DNA polymerase. PCR reactions (total volume 50 μl) were performed in a GeneAmp PCR System 2400 temperature cycler (Perkin Elmer), starting with 2 min at 96°C followed by 35 cycles of 96°C for 30 s, 55°C for 30 s and 72°C for 1 min. Primers used were: DAGMH (forward) 5′-GGAGAACCCAACCAGCGCCCAGAGC-3′; DAGAH (reverse) 5′-CGGGTGATATTCTGCAGGGTGATGG-3′; DAGAB (forward) 5′-CCATCACCCTGCAGAATATCACCCG-3′; HP20 (reverse) 5′-GACCGGTATGGGGTCATGTT-3′. Agarose gel electrophoresis was used to analyse the PCR experiments and DNA products were identified for the presence of 0.5 μg/ml ethidium bromide. The DNA 1 kb Plus Ladder™ was purchased from Gibco and the control primer set for human β-actin from Stratagene.

3. Results and discussion

SDS–PAGE Western blot experiments were carried out using a commercially available antibody (IgG) (clone 8D5, mouse monoclonal from Novocastra Laboratories) directed versus the last C-terminal 15 amino acids of β-dystroglycan. In Fig. 1 is reported a schematic drawing of the dystroglycan molecule showing the epitope under analysis.

As shown in Fig. 2A, β-dystroglycan was detected as the common 43 kDa band in the HC11 normal mouse and in the 184B5 non-tumorigenic human mammary gland cell lines whereas in the breast cancer cell lines (T47D, BT549, MCF7) a band displaying a reduced molecular mass (≈31 kDa) was also clearly detectable. Remarkably, in the MCF7 cell line, this band was the only one revealed by the anti-β-dystroglycan antibody. In order to rule out the possibility of artefacts due to the sample preparation, we have used two different total protein extraction procedures (see Section 2), and we have obtained similar results (data not shown). It is noteworthy that a series of independent protein extraction-blot experiments were carried out on each of the cell lines under analysis, always obtaining the same band pattern. In addition, single cell lysates were repeatedly used after many cycles of freezing/thawing and gave similar results, thus indicating that an eventual activation of proteases during sample preparation is very unlikely.

We have also found the β-dystroglycan ≈31 kDa band in a number of different cell lines from human colon (HT29, HCT116 and SW620), prostate (DU145) and cervical (HeLa) cancer (Fig. 2B), indicating that the phenomenon is not restricted to breast cancer cells. Interestingly, using a monoclonal anti-α-dystroglycan (VIA4-1) antibody, we were unable to detect the α-dystroglycan in the MCF7 and DU145 cells that only expressed the aberrant ≈31 kDa band (Fig. 2C). The α-dystroglycan band was also undetectable in HT29 that expressed both the 43 and the ≈31 kDa β-dystroglycan band (Fig. 2C), suggesting also in this case a dramatic alteration of the entire dystroglycan complex. To further extend our analysis, we also analysed the expression of β-dystroglycan in a series of seven NMU-induced rat mammary tumours and found that most of them express the aberrant ≈31 kDa band (Fig. 2D).

It is noteworthy that the VIA4-1 antibody recognises an α-dystroglycan carbohydrate epitope. Therefore, we cannot rule out the possibility that VIA4-1 antibody fails to recognise α-dystroglycan in tumour cells due to an alteration of its glycosylation pattern. To verify whether α-dystroglycan was released in the culture medium, we have carried out Western blot analysis on 24 h conditioned media samples but we were unable to detect any signal in both normal and tumour cells despite the fact that we concentrated the media up to 100-fold (data not shown).

In order to exclude the possibility that the abnormalities detected in the dystroglycan molecule could depend on alternative splicing and/or expression of eventual dystroglycan isoforms, we carried out qualitative RT-PCR experiments using total RNA extracted from most of the cell lines under analysis. In Fig. 3A is reported a scheme of the human dystrogly-

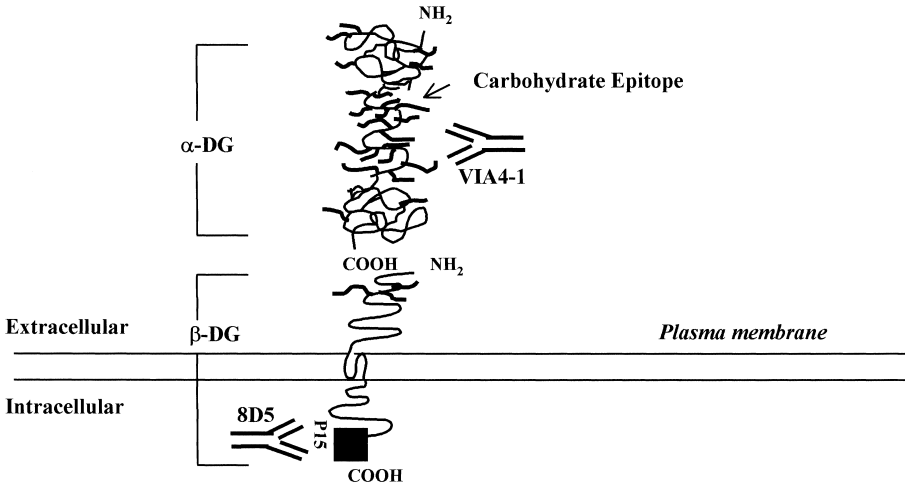

Fig. 1. Schematic drawing of dystroglycan. The dystroglycan portions recognised by the antibodies used for Western blot experiments are depicted. The mouse monoclonal VIA4-1 recognises a carbohydrate epitope belonging to α-dystroglycan. The 8D5 mouse monoclonal recognises the very last 15 amino acids (P15) belonging to the C-terminus of β-dystroglycan.

can cDNA sequence. Dystroglycan human gene has been sequenced and mapped to chromosome 3 and displays an uncomplicated structure, sharing a single intron within its 5′ end [26]. We used a RT-PCR approach to analyse a region that covers the last part of the C-terminal region of α-dystroglycan and almost the entire β-dystroglycan sequence in normal and malignant mammary gland cells. As shown in Fig. 3A, two different pairs of primers were used in order to amplify a sequence of 485 bp (corresponding to the C-terminal region of α-dystroglycan, next to the Gly-Ser$^{653-654}$ post-translational proteolytic site) and a sequence of 731 bp (almost corresponding to the whole sequence of β-dystroglycan). As reported in Fig. 3B, a number of bands were obtained whose size exactly matches the expected calculated lengths (see Fig. 3A) in the 184B5, MCF7 and DU145 cell lines. Using most of the cell lines under analysis, similar results were obtained (data not shown). These data indicate that the ≈31 kDa β-dystroglycan protein does not originate by alternative RNA processing and/or expression of isoforms.

Malignant cell lines frequently overexpress proteases [27], especially membrane-associated metalloproteases [28–30], that are responsible for the extracellular matrix degradation, facilitating tumour invasiveness. It is possible that one of these proteases provokes (or enhances) the degradation of β-dystro-

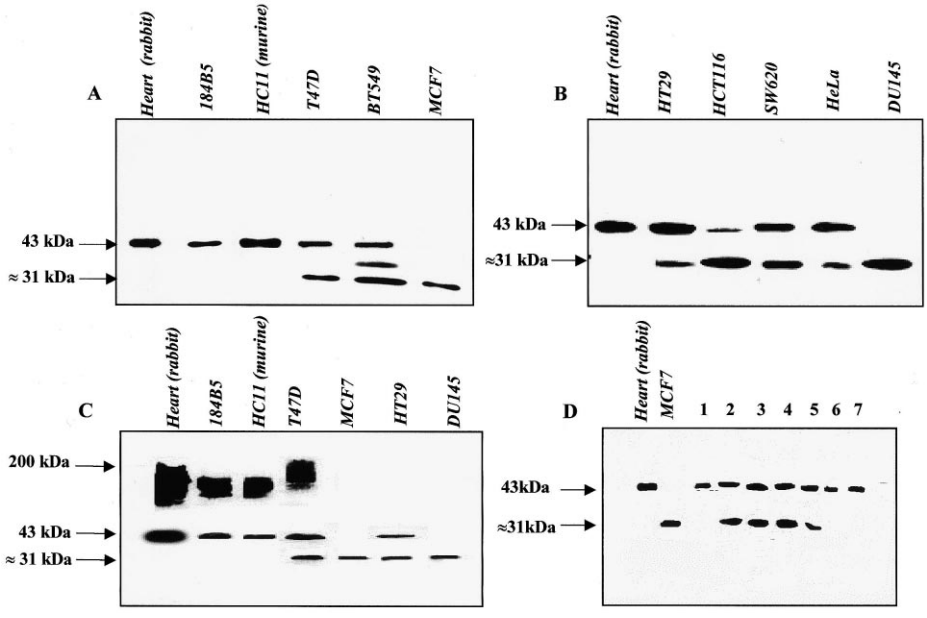

Fig. 2. Western blot analysis. A: β-Dystroglycan is recognised by the monoclonal antibody 8D5 (see Fig. 1). Unless indicated, all the experiments refer to human cell lines. The bands at 43 and ≈31 kDa are indicated by arrows. An intermediate band is also present in BT549 carcinoma cell line. B: β-Dystroglycan is recognised by the monoclonal antibody 8D5 (see Fig. 1). The bands at 43 and ≈31 kDa are indicated by arrows. C: α-Dystroglycan and β-dystroglycan are recognised by mouse monoclonal antibodies VIA4-1 and 8D5, respectively (see Fig. 1). Molecular mass marker at 200 kDa as well as the bands at 43 and ≈31 kDa are indicated by arrows. The nitrocellulose membrane was first incubated with anti-α-dystroglycan and after with anti-β-dystroglycan antibody. D: β-Dystroglycan is recognised by the monoclonal antibody 8D5 (see Fig. 1). The bands at 43 and ≈31 kDa are indicated by arrows. Numbers from 1 to 7 show a series of NMU-induced rat mammary tumours.

glycan. If this would be the case, the breakdown should take place at the N-terminus of β-dystroglycan [7], since the 8D5 antibody recognises a C-terminal epitope of the molecule (see Fig. 1). A proteolytic process involving the removal of a certain amount of the N-terminal portion of β-dystroglycan might affect the interaction between α- and β-dystroglycan. In fact, we have recently demonstrated by recombinant expression and solid-phase binding assays that this region is able to bind with high affinity and independently from glycosylation to the C-terminal region of α-dystroglycan [31]. Our observation that the α-dystroglycan is not detectable in the cell lines (i.e. MCF7 and DU145, see Fig. 2C) that only express the β-dystroglycan ≈31 kDa band is consistent with this hypothesis (see above).

Loss of a large portion of the N-terminus of the β-dystroglycan molecule might also compromise its targeting into the cell membrane. To verify this possibility, we have carried out protein extraction experiments at low-ionic strength (in 20 mM HEPES, pH 7.4, in the absence of any detergent, see Section 2) which allow to separate cytoskeletal and membrane-bound from cytosolic proteins. We found that the β-dystroglycan bands were present in the membrane-bound fraction of the BT549 cell line (Fig. 4). In addition, we were not able to resolubilise the ≈31 kDa β-dystroglycan band using KCl as expected for a cytoskeleton-associated protein (Fig. 4). Thus, all the protein bands detected by the 8D5 antibody harbour their transmembrane segment, supporting the hypothesis that β-dystroglycan undergoes a proteolytic cleavage within its N-terminal extracellular region that does not affect its targeting into the cell membrane. The alternative

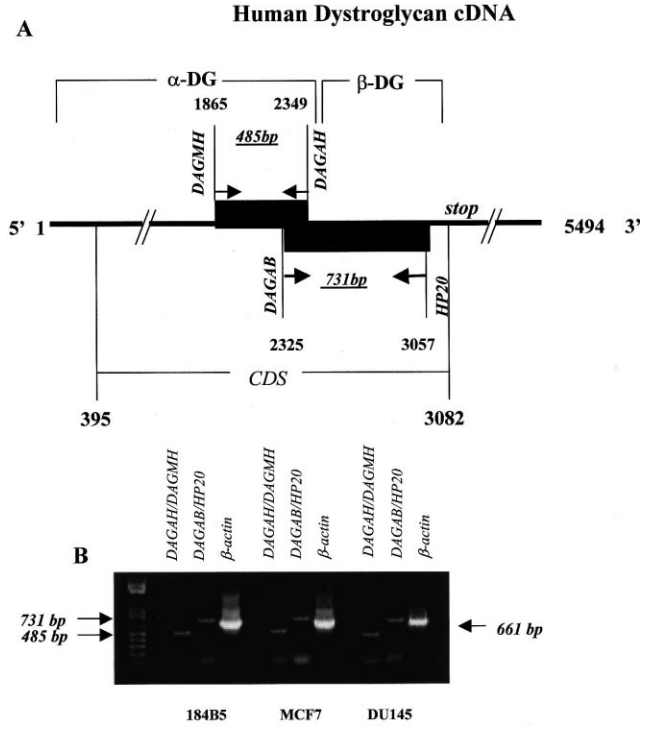

Fig. 3. RT-PCR analysis. A: Scheme of the human dystroglycan cDNA sequence. The primers used and the size of the amplified portions, matching coding derived sequence (CDS) portions belonging to α- and β-dystroglycan, are reported. B: 1% Agarose gel showing DNA bands originating from PCR amplification experiments referring to different cell lines. Control bands (661 bp) obtained using human β-actin primers are also included.

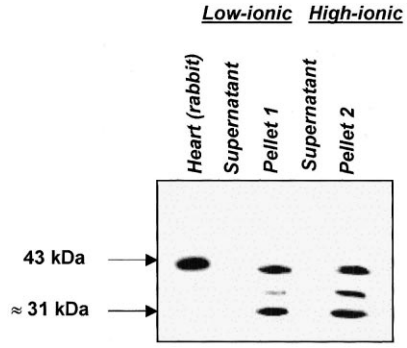

Fig. 4. Western blot of protein extracts at low- and high-ionic strength. BT549 human breast cancer cell extracts: pellet and supernatant fractions were analysed using anti-β-dystroglycan antibody. Pellet 1 was resuspended in TX-100 lysis buffer for Western blot analysis. A separate pellet was washed and resuspended in high-ionic conditions. Pellet 2, originating from high-ionic strength extraction, was also resuspended in TX-100 lysis buffer (see Section 2). The bands at 43 and ≈31 kDa are indicated by arrows, an intermediate band is also present (see Fig. 2A).

possibility that the proteolysis could take place at the level of the cytosolic region, and that the ≈31 kDa protein would be recovered in the insoluble fraction because of its association to cytoskeletal proteins, is challenged not only by the experimental results reported in Fig. 4 but also by the notion that the entire β-dystroglycan cytoplasmic tail has a calculated molecular mass of about ≈13.5 kDa. It is unlikely that a putative protein fragment of this size would show such an anomalous electrophoretic behaviour accounting for a ≈31 kDa molecular mass. In order to collect conclusive evidences on the hypothesis of a proteolytic breakdown, taking place within the β-dystroglycan extracellular region will be necessary to purify the ≈31 kDa peptide and unambiguously identify its N-terminal amino acids. In fact, it cannot be completely ruled out that the reduction of β-dystroglycan size we observed might originate by a reduction (or lack) of the carbohydrate epitopes modifying its extracellular region [6,7]. The decrease or absence of glycosylation may directly reduce the molecular mass of β-dystroglycan. However, the hypothesis that the ≈31 kDa band would originate by a significant reduction of β-dystroglycan glycosylation is challenged by the following considerations. In the extracellular region of human dystroglycan there is only one N-linked glycosylation site (Asn661). Recent experiments by Campbell's laboratory, in which tunicamycin and PNGaseF were employed, show that N-linked glycosylation of β-dystroglycan accounts only for ≈5 kDa [37]. Two candidate O-linked glycosylation sites are also present, Ser729 and Thr734 (based on the *NetOGlyc* program analysis available at the *ExPASy* server, Geneva, Switzerland), but there are not direct evidences indicating the presence (and the eventual size) of O-linked carbohydrate molecules. It should also be noted that in two genetic variants of the C2 muscle cell line, S26 and S27 which show a defect in glycosaminoglycan biosynthesis, no alteration of *Torpedo californica* β-dystroglycan electrophoretic mobility was detected [38]. Last but not least, the anomalous electrophoretic behaviour of β-dystroglycan recombinant protein fragments [28–32] strongly suggests that the difference between the calculated (26.4 kDa) and apparent molecular mass (43 kDa) of β-dystroglycan does not depend only on the presence of its carbohydrate fraction. [28]. Taken together, these results indicate

that the reduction of β-dystroglycan molecular mass (≈31 kDa) is very unlikely to depend (at least entirely) on altered glycosylation, thus favouring the hypothesis of a protease-mediated cleavage.

Interactions between cell surface receptors and extracellular matrix binding partners play crucial roles starting from early morphogenesis as well as during adulthood [33–35]. In particular, the gene disruption experiment has revealed a crucial role for dystroglycan since mouse development was arrested as early as day 6.5 [36]. Also during tumorigenesis a critical role has been assigned to the interactions between the cells and the surrounding matrix. For example, the expression of several integrin receptors is altered in malignant cells as compared to their normal counterparts [21], and integrins have been reported to play an important role for cell growth and metastasis of several human tumours [20–22]. In this context, the finding that dystroglycan is present in an altered form in several carcinoma cell lines is relevant. A reduction (or abolishment) of dystroglycan function could influence the formation of strong contacts between basement membranes and the cytoskeleton of cells [18], thus eventually favouring tumour development and invasiveness. It is also of interest that β-dystroglycan has been reported to bind to the growth factor-associated adaptor protein Grb2 [16] and recently, it was demonstrated that the focal adhesion kinase $p125^{FAK}$ in brain synaptosomes is associated to the dystrophin–glycoprotein complex [19]. The alteration of dystroglycan found in carcinoma cell lines might have dramatic consequences on signal transduction and consequently on the metabolism and growth rate of tumorigenic cells.

It was recently reported that in several cell lines (CHO, RT4 schwannoma and bovine aortic endothelial) a certain fraction of α-dystroglycan is recovered as soluble in the culture medium [37]. As already mentioned, degradation of the extracellular region of β-dystroglycan would liberate α-dystroglycan from its membrane-bound location. Our data may suggest that α-dystroglycan is indeed not found at its normal membrane-associated location in some of the carcinoma cell lines which were analysed (Fig. 2C). The interaction between α- and β-dystroglycan is supposed to have a high affinity, and in vitro a medium–high affinity K_d of about 10 μM was estimated [31]. However, other factors, like the presence of stabilising proteins (for example members of the sarcoglycan family), influence the stability of the dystroglycan complex in epithelial cells [31,38].

The present data provide evidence that dystroglycan can be processed in an aberrant fashion in several carcinoma cell lines belonging to different tissues. Further experiments will be necessary to verify whether abnormalities within dystroglycan processing mechanisms play a specific role during carcinogenesis in vivo and might have clinically relevant implications.

Acknowledgements: We gratefully acknowledge Jürgen Engel (Basel), Markus A. Ruegg (Basel) and John L. Muschler (Berkeley) for useful suggestions and critical reading of the manuscript and Mr. Pietro Quirini (Rome) for his technical assistance.

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