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Biochimica et Biophysica Acta 1416 (1999) 155–160

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Identification of 130 kDa cell surface LDL-binding protein from smooth muscle cells as a partially processed T-cadherin precursor

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Received 17 July 1998; received in revised form 3 November 1998; accepted 3 November 1998

Abstract

Atypical cell surface lipoprotein-binding proteins of 105 kDa and 130 kDa are present in membranes of vascular smooth muscle cells. We recently identified the 105 kDa protein from human aortic media as T-cadherin, an unusual glycosylphosphatidylinositol (GPI)-anchored member of the cadherin family of cell adhesion proteins. The goal of the present study was to determine the identity of 130 kDa lipoprotein-binding protein of smooth muscle cells. We applied different approaches that included protein sequencing of purified protein from human aortic media, the use of human T-cadherin peptide-specific antisera, and enzymatic treatment of cultured cells with trypsin and GPI-specific phospholipase C. Our results indicate that the 130 kDa protein is a partially processed form of T-cadherin which is attached to the membrane surface of smooth muscle cells via a GPI anchor and contains uncleaved N-terminal propeptide sequence. Our data disclose that, in contrast to classical cadherins, T-cadherin is expressed on the cell surface in both its precursor (130 kDa) and mature (105 kDa) forms. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Lipoprotein; Cadherin; Vascular smooth muscle cell

1. Introduction

We have previously demonstrated the existence of atypical lipoprotein-binding proteins in vascular smooth muscle cells (SMC) and hypothesized that they may represent signalling-coupled receptors for lipoproteins [1]. Two variant forms of these proteins of 105 kDa and 130 kDa (p105 and p130) were usually present in the same cell type although the ratio of these proteins varied between SMC of different origin (ex vivo or in culture, human or rat). Ligand-binding studies have shown that p105 and p130 possess apparently identical ligand selectivities that are clearly distinct from those of all other recognized lipoprotein receptors [2]. In order to molecularly identify these atypical lipoprotein-binding proteins we developed a procedure for their purification from human aortic medial tissue. Sequencing of the purified 105 kDa form (which is predominant in human aortic media) revealed its identity as T-cadherin,

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PII: S0005-2736(98)00218-1

an unusual GPI-anchored member of the cadherin family of cell adhesion molecules [3]. The goal of the present study was to identify the 130 kDa lipoprotein-binding protein of SMC, and we present data demonstrating that p130 is a partially processed (precursor) form of T-cadherin.

2. Materials and methods

2.1. Materials

Electrophoresis reagents were obtained from Bio-Rad (Hercules, USA). Delipidated powdered milk, Rapilait, was from Migros (Basel, Switzerland). Trypsin and cell culture reagents were from Gibco (Life Technologies, Basel, Switzerland). CHAPS, buffers and other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Generation of anti-T-cadherin peptide antibodies

Peptides corresponding to human T-cadherin (cadherin-13 [4], GenBank accession No. L34058) precursor amino acid positions 140–160, 161–179, 260–271 were prepared by solid phase synthesis on Applied Biosystems 431A Synthesizer using Fmoc-amino acyl polymers (Bachem, Switzerland). Conjugation of peptides, immunization of animals, characterization of antisera and purification of IgG fraction were performed as described previously [3].

2.3. Cell culture and treatment of cells with trypsin and GPI-specific phospholipase C

Human aortic smooth muscle cells (hSMC) were isolated, phenotypically characterized and propagated as described [5]. For the studies herein, hSMC were used between passages 4 and 18 and normally cultured in Dulbecco's medium containing 5 mM glutamine, 10 mM TES-NaOH, 10 mM HEPES-NaOH, pH 7.3, 0.05 mg/ml gentamicin and 10% fetal calf serum (FCS). Serum-free medium (SF) was minimal essential medium containing Earle's salts and all ingredients as given above with the exception that serum was substituted with 0.1% (w/v) BSA. Prior to experimental protocols hSMC were maintained for 48 h under serum-free conditions (0.1% BSA replacing 10% FCS). For determining sensitivity to PI-PLC hSMC were washed once with PBS, once with 0.01 N NaOH, pH 11.0, to remove peripheral membrane proteins, and once again with PBS. hSMC monolayers were then incubated at 37°C for 90 min in incubation buffer (50 mM Tris-HCl, pH 7.6, 0.15 mM $CaCl_2$, protease inhibitors) without (control) or with inclusion of PI-PLC (5.0 U/ml; ICN Biomedicals). The culture supernatants were collected, freed of cellular debris by centrifugation and concentrated 50-fold by ultrafiltration. Cell layers were washed with PBS before lysis in 1% SDS/1 mM PMSF. For determining trypsin sensitivity hSMC were washed with HBSS containing 5 mM $CaCl_2$ and then incubated for 30 min at 37°C in the same buffer without (control) or with addition of 0.25% trypsin. Incubations were terminated by addition of 1% soybean trypsin inhibitor, and cells were washed with HBSS containing 0.1% trypsin inhibitor before lysis in 1% SDS/1 mM PMSF. Cell lysates were analysed for both LDL binding (ligand blotting, with biotinylated LDL as ligand) and T-cadherin immunoreactivity (immunoblotting, using human anti-T-cadherin peptide antiserum).

2.4. Purification and sequencing of p130 from human aortic media membranes

LDL-binding protein p130 was purified from human aortic medial tissue exactly as described for the purification of p105 [3] except for the final electrophoretic steps. After SDS-gel electrophoresis the 130 kDa band was excised, the protein was electroeluted and subjected to isoelectrofocussing in a slab gel containing 2% Ampholines (pH 3–10). The protein detected by Coomassie/water stain was electroeluted, electrophoresed again in SDS-gel and transferred to PVDF membrane which was finally processed for protein sequencing as described previously [3].

2.5. Lipoprotein preparation, ligand and immunoblotting

LDL (density 1.019–1.063 g/ml) were isolated from the plasma of healthy male humans using sequential buoyant density centrifugation techniques with use of NaBr for density adjustments [6]. Biotinylated

LDL was prepared by incubation of LDL (2 mg/ml) with D-biotin-*N*-hydroxysuccinimide (0.3 μmol/2 mg LDL) and subsequent extensive dialysis [7]. Ligand (LDL) binding procedures have been fully described and validated previously [1,2]. Briefly, SDS-polyacrylamide (8%) gel electrophoresis was performed under non-reducing conditions, proteins were electrophoretically transferred onto nitrocellulose membranes, and after blocking of non-specific binding blots were sequentially incubated with biotinylated LDL (80 μg/ml) and streptavidin-horseradish peroxidase (HRP) conjugate. For immunodetection of T-cadherin proteins were electrophoresed and electroblotted as above, and blots were sequentially incubated with polyclonal anti-T-cadherin peptide antisera and anti-rabbit IgG-HRP conjugate. TBS containing 2% (w/v) milk was used for incubations/washing procedures with LDL, anti-T-cadherin antibodies, and IgG-HRP conjugate. TBS without milk was used during incubations with streptavidin-HRP conjugate. For both ligand and immunoblots the Amersham ECL system was used for detection, and a co-electrophoresed biotinylated molecular weight standard mixture (Bio-Rad), consisting of ovalbumin (45 kDa), serum albumin (66 kDa), phosphorylase *b* (97 kDa), β-galactosidase (116 kDa) and myosin heavy chain (205 kDa) was used for calibration.

3. Results and discussion

We have previously used ligand-blotting techniques to characterize the binding properties of two atypical lipoprotein-binding proteins of 105 kDa and 130 kDa present in membrane preparations from human aortic media and from cultured human and rat aortic SMC [1,2]. The identity of p105 as the cell adhesion glycoprotein T-cadherin was determined after its purification from human aortic medial tissue [3]. Although the nature of the 130 kDa protein was unknown a striking similarity of ligand-binding characteristics displayed by p105 and p130 [2] was thought to be predictive of a connected identity. Moreover, in cultures of SMC their expression levels are synchronously regulated in response to changes in growth conditions. For example, p105 and p130 protein levels concomitantly increase during quiescence and concomitantly decline after addition of serum or individual growth factors [8]. Similarly, activation of specific second messenger systems in SMC results in parallel modulation of expression levels of the two proteins [9].

The above data, however, provide only indirect evidence of relatedness between p105 and p130. In order to more directly ascertain whether p105 and 130 are related proteins, in this study we first tested their immunoreactivity to antisera generated against three synthetic T-cadherin fragments [3]. All three peptide antisera recognized both p105 and p130 (Fig. 1), indicating that the two proteins have identical fragments of primary structure. In the presence of Ca^{2+}, mature T-cadherin is known to be protected from trypsin digestion [10]. To investigate whether p130 may be a precursor of the p105 protein, hSMC monolayers were treated with trypsin (in the absence or presence of Ca^{2+}) and thereafter residual

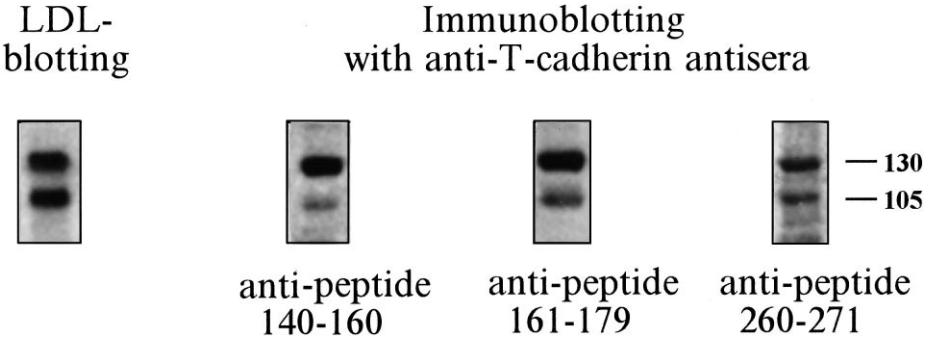

Fig. 1. Recognition of 105 kDa and 130 kDa lipoprotein-binding proteins by antisera to synthetic T-cadherin fragments. hSMC lysates were electrophoresed in 8% SDS-PAGE and electroblotted onto nitrocellulose membrane. Blots were analysed by ligand blotting with LDL or by immunoblotting with polyclonal antibodies against synthetic peptides corresponding to T-cadherin precursor amino acid positions 140–160, 161–179 and 260–271.

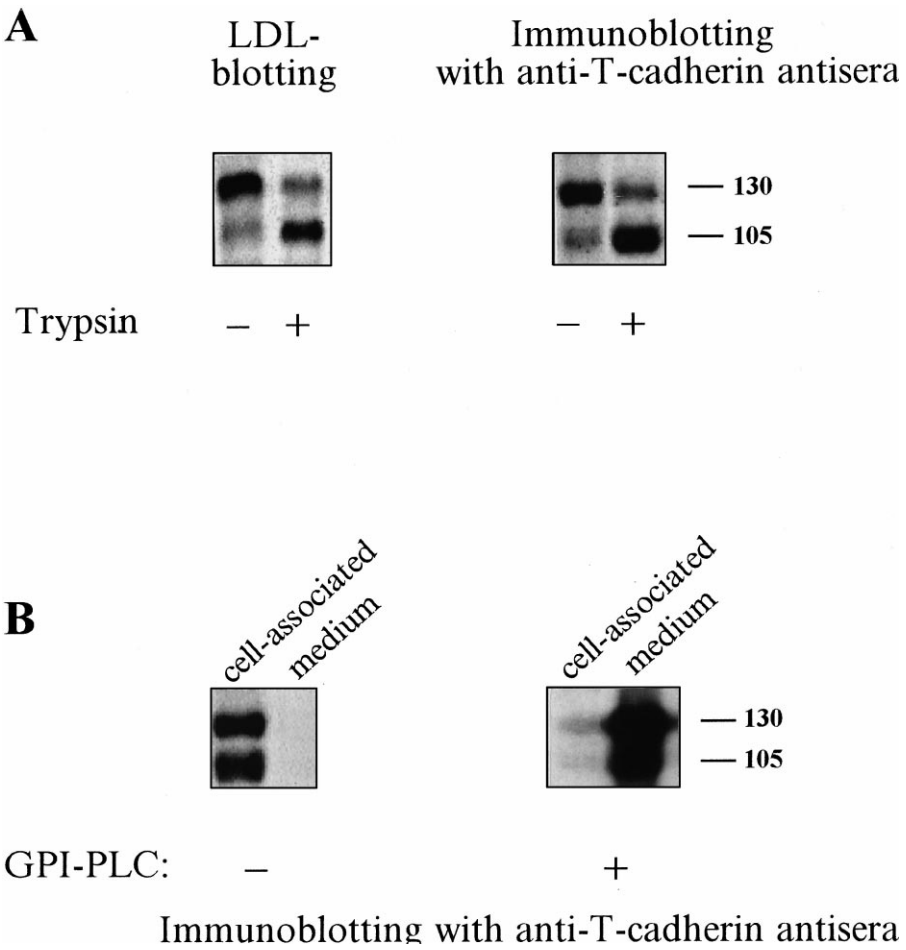

Fig. 2. Sensitivity of cell surface p105/p130 to treatment with trypsin or GPI-specific phospholipase C. Cultured hSMC were treated with trypsin/Ca^{2+} (A) or GPI-specific phospholipase C (B) as described in Section 2. Thereafter whole cell lysates were analysed by ligand blotting with LDL or immunoblotting with antibody against T-cadherin peptide 140–160. In panel B, media supernatants were also collected, concentrated and analysed for T-cadherin peptide (140–160) immunoreactivity.

cell-bound p105 and p130 determined. In the absence of Ca^{2+} both p105 and p130 are sensitive to trypsin digestion, whereas in the presence of Ca^{2+} there was a selective loss of p130 and simultaneous accumulation of p105 (Fig. 2A). Thus, p105 appears to be a trypsinolytic product of p130, and since the newly formed p105 remained cell-bound, it is most likely that trypsin cleaves a peptide from p130 at a distal domain of the protein.

Consistent with the recognition of T-cadherin as a GPI-anchored protein [11] and the cellular compartment localization of several other GPI-anchored proteins [12,13] we have recently demonstrated enrichment of p105/T-cadherin in a minor caveolin-rich membrane fraction of SMCs [14]. We also demonstrated co-distribution of p130 protein in this 'caveolar' fraction [14]. Therefore to determine whether p130 is also a GPI-anchored protein, hSMC were treated with GPI-specific phospholipase C (PI-PLC) and both culture supernatant and cell layers examined for T-cadherin immunoreactivity. PI-PLC rendered p105 and p130 soluble, thus demonstrating that both proteins were anchored to the membrane by means of the GPI group (Fig. 2B). The above data further support the possibility that p130 may be a precursor of the mature 105 kDa T-cadherin protein of SMC, and concur with previous observations made for T-cadherin precursor in CHO cells transfected with chick T-cadherin [10].

In order to identify p130 unequivocally, we purified the protein to homogeneity according to procedures previously developed for purification of p105

protein [3]. In all steps excepting gel electrophoresis p130 co-purified with p105 (data not shown). Sequencing of a proteolytic fragment of p130 produced the following sequence: E-V-x-x-P-Y-F. Sequence analysis using the GenBank data has shown that the sequence corresponds to the amino acid residues 67–73 of the putative propeptide region of the human T-cadherin precursor [4]. Although limited, these sequencing data provide additional evidence that p130 may be a partially processed (precursor) form of T-cadherin.

This study has obtained several lines of evidence supporting that p130 is a precursor of p105. First, trypsin treatment of cells in the presence of Ca^{2+} produced a shift between the levels of two proteins, namely a simultaneous decrease in p130 and accumulation of p105. This shift in the relative levels of p105 and p130 probably arises because trypsin cleavage sequences (Arg-X, Lys-X) are present at the amino-terminal end of T-cadherin propeptide. Cleavage does not proceed further since mature T-cadherin is resistant to mild trypsinolysis in the presence of Ca^{2+} [11]. Second, both proteins were recognized on Western blots by three different synthetic T-cadherin peptide antisera. Importantly, one of these antisera was targeted to residues 140–160 of T-cadherin precursor, these residues corresponding to the amino acid residues 2–22 of the putative mature protein [4]. Data obtained following trypsin/Ca^{2+} treatment of intact hSMC might therefore be interpreted to indicate a restricted cleavage of T-cadherin precursor (i.e. cleavage of the amino-terminal propeptide) with resultant production of mature T-cadherin. Third, partial sequencing data demonstrated that p130 contains a fragment of the propeptide sequence of human T-cadherin, and fourth, GPI-specific phospholipase C rendered both proteins soluble. Together these results would indicate that p130 is a partially processed form of T-cadherin in which the N-terminal propeptide has not been cleaved, and that both this and the mature forms of T-cadherin are attached to the cell surface membrane via a GPI anchor. This surface attachment of precursor and mature T-cadherin proteins contrasts with classical cadherins. Classical cadherins are processed intracellularly and their precursors are inactive in homophilic binding [15].

The gene for T-cadherin, like other cadherins encodes for a pro-protein. Processing of T-cadherin has not been studied in detail, but by analogy with what is known for classical cadherins and GPI proteins [16,17] one may propose that it includes three steps. First, a hydrophobic NH_2-terminal signal peptide directing nascent T-cadherin to the ER is removed by signal peptidase. Second, the COOH-terminal peptide is removed with simultaneous attachment of the GPI moiety. Finally, the NH_2-terminal propeptide containing protease processing signal sequence, RQKR, at the COOH terminus, is cleaved, thus producing mature T-cadherin. In this study we nominate p130 as a 'partially processed' T-cadherin precursor because it is processed at the C terminus (our data on GPI-phospholipase treatment clearly indicate that p130 is a GPI-attached protein) but not at the N terminus, where the signal peptide is removed, but the propeptide sequence remains uncleaved.

In conclusion, therefore, we have identified the 130 kDa lipoprotein-binding protein as a partially processed T-cadherin precursor and demonstrate for the first time that T-cadherin can be expressed on the surface of vascular smooth muscle cells as both mature and precursor forms. Further studies are required in order to determine the specific role of the precursor molecule of T-cadherin in cell adhesion and communications.

Acknowledgements

This work was supported by grants from the Swiss National Foundation (Nos. 31-41874.94 and 31.52290.97), the Swiss Heart Foundation, the Russian Foundation for Fundamental Research (No. 95-04-12253) and INTAS (No. 93-3260).

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