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Identification of an atypical lipoprotein-binding protein from human aortic smooth muscle as T-cadherin

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Abstract We have previously described an atypical lipoprotein-binding protein of about 105 kDa (p105) in membranes of vascular smooth muscle cells (VSMCs) that is distinct from currently known lipoprotein receptors. In the present work we have developed a procedure for purification of p105 from human aortic media. Partial sequencing of purified protein has revealed identity of p105 with human T-cadherin. Anti-peptide antisera raised against human T-cadherin recognized a protein spot corresponding to the purified p105 on two-dimensional Western blots. The antisera also inhibited LDL binding to p105 on ligand blots. We conclude that the 105 kDa lipoprotein-binding protein present in human VSMCs is T-cadherin, an unusual glycosylphosphatidylinositol-anchored member of the cadherin family of cell-cell adhesion proteins.

Key words: Lipoprotein; Cadherin; Vascular smooth muscle cell

1. Introduction

During the last decade the understanding of both cellular effects of lipoproteins and mechanisms of cell-lipoprotein interactions has significantly broadened. It is increasingly recognized that, quite apart from their lipid-transporting functions, lipoproteins can stimulate a number of cellular metabolic events by apparently endocytosis-independent mechanisms. In particular, lipoproteins activate second messenger systems, regulate gene expression and stimulate proliferation in vascular smooth muscle cells (VSMCs) [1–5]. It has been hypothesized that these processes are mediated by membrane receptor(s) [1,6] that are distinct from known lipoprotein receptors [7,8]. We have more recently conducted a search for candidate signalling receptors in VSMCs using a method of ligand blotting that exploits the property of lipoprotein receptors to retain high affinity and selectivity for their ligands on Western (ligand) blots. Ligand blotting has been widely and successfully applied for investigation of ligand and inhibitor selectivity of apo B,E (LDL) receptor [9], VLDL receptor [10], LRP [11], gp330/megalin [12], scavenger receptor [13], lipolysis-stimulated receptor [14] and HDL-binding proteins [15]. Application of this technique for characterization of lipoprotein-binding proteins in VSMC membranes resulted in detection of a major lipoprotein-binding protein of 105 kDa (p105) and a minor 130 kDa lipoprotein-binding protein (p130) [16]. The ligand selectivity and binding properties of p105 and p130 are apparently identical and clearly distinct from those of all other recognized lipoprotein receptors [17]. The identity of these atypical lipoprotein-binding proteins was not known. We have accordingly developed an original procedure for purification of p105 from human aortic media and determined its partial amino acid sequence. This paper describes the scheme of purification of p105 and presents data indicating that p105 is identical with T-cadherin, a protein belonging to the cadherin family of cell-cell adhesion proteins [18,19].

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Abbreviations: VSMC, vascular smooth muscle cell; LDL, low density lipoprotein; mAb, monoclonal antibody; IEF, isoelectric focusing; 2-D, two-dimensional; GPI, glycosylphosphatidylinositol

2. Materials and methods

2.1. Materials

Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, USA). Defatted dry milk, Rapilait, was from Migros (Basel, Switzerland). CHAPS, iminodiacetic acid-agarose, Ampholines Ph 3.5–10, CNBr-activated Sepharose 4B, protein A-agarose, buffers and other chemicals were purchased from Sigma Chemical Co. (St. Louis, USA).

2.2. Ligand and immunoblotting

The method of ligand (LDL) blotting has been fully described previously [16]. Briefly, protein samples were solubilized in Laemmli buffer without reducing agents, electrophoresed on 8% SDS-polyacrylamide gels and electroblotted onto nitrocellulose. Nitrocellulose membranes were pre-blocked in TBSM (5% solution of defatted dry milk in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) and incubated at room temperature in TBSM containing LDLs (100 μg protein/ml, unless otherwise stated). After washing with TBSM, the membranes were incubated with affinity-purified anti-LDL antibodies conjugated to peroxidase, washed again and stained by the Ni^{2+}-diaminobenzidine method [20]. Quantitative estimates of LDL binding were obtained by scanning of blots (Hewlett Packard Scanjet 4P) and use of SigmaGel software. Immunoblotting procedures were essentially the same except that detection of bound primary antibodies was performed using goat anti-rabbit IgG antibodies coupled to peroxidase.

2.3. Purification of p105 from human aortic media membranes

Microsomal membranes were isolated from human aortic media as described before [21,16]. The membranes were solubilized in buffer A (20 mM Tris, 5 mM EDTA, 0.2 mM PMSF, pH 8.0) containing 10 mM CHAPS (60 min on ice with stirring) and centrifuged at $100\,000 \times g$ for 1.5 h. The supernatant was applied to a column of DEAE-Toyopearl (5 mg protein/ml gel). The column was washed with two volumes of buffer A, one volume of buffer B (20 mM MOPS, 10

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mM CHAPS, 0.2 mM PMSF, pH 7.2) and then eluted with 3 volumes of buffer B containing 0.5 M NaCl. The eluate was passed over a column of immobilized iminodiacetic acid (2 mg protein/ml gel) which had been loaded with Zn^{2+} ions according to the manufacturer's protocol, and the column was washed with buffer B containing 0.5 M NaCl. The flowthrough and the wash were combined and concentrated by ultrafiltration under the pressure of nitrogen, and then passed through a column of Sepharose 4B containing immobilized mAb clone 9B8 (see below). The flowthrough was loaded onto SDS-containing 8% polyacrylamide gel (4 mg protein per 13.5 cm×0.75 mm slab) and electrophoresed [22]. The gels were stained with 0.05% Coomassie R-250 water solution, destained in water, and the band containing p105 (identified by comparison with a ligand blot processed in parallel) was excised. The protein was electroeluted from the gel (Model 422 Electro-Eluter, BioRad), concentrated by ultrafiltration and applied to the first dimension isoelectrofocusing gels (tubes 3 mm×8 cm, 5% PAAG, 8 M urea, 2% Triton X-100, 2% Ampholines pH 3.5–10.0). Isoelectrofocusing was performed at 400 volts overnight, then at 800 volts for 2 h. The tube gels were then fixed with agarose on top of 8% SDS-polyacrylamide slab gels and electrophoresed in the Laemmli buffer system. The protein spots were detected by Coomassie staining as described above, excised and electroeluted. After testing an aliquot for purity by analytical 2-D gel electrophoresis, the samples were again electrophoresed in 8% SDS-polyacrylamide gel and transferred onto PVDF membrane by electroblotting [23]. The blots were stained with 0.2% Coomassie in 50% methanol and destained in 50% methanol; the protein spots were excised, dried under the stream of nitrogen and stored in Eppendorf tubes until sequencing. The purification procedure consistently yielded ≈10 μg p105 per 100 mg of starting membrane protein extract.

2.4. Generation of 9B8 monoclonal antibody

This clone was selected after immunization of BALB/c mice with partially purified p105. Fusion was performed according to [24] using mouse myeloma X63-Ag8.653 cell line and PEG 1500. Screening of mAbs was carried out by a dot-blot procedure using antigen adsorbed on nitrocellulose membrane. The selected hybridoma was cloned and then grown in the peritoneal cavity of BALB/c mice. The mAb was precipitated from ascitic fluid by ammonium sulfate, further purified by protein A-chromatography and immobilized on CNBr-activated Sepharose 4B.

The 9B8 mAb did not recognize p105, but interacted with an unknown protein having both a molecular weight and pI very close to those of p105. This immunoaffinity step eliminated the 9B8 antigen which partially overlapped and interfered with further purification of p105 from two-dimensional gels.

2.5. Sequencing of p105

Sequencing of p105 was performed in the Laboratory of Protein Chemistry, Department of Biochemistry (Prof. Paul Jeno), Biocenter, Basel, Switzerland. Peptide fragments of p105 were generated by trypsin digestion of the protein on PVDF membranes, separated by reversed phase HPLC and subjected to gas phase sequencing. Matching of peptide amino acid sequences in the SWISS-PROT database using DNA SUN software revealed identity of the fragments with human T-cadherin (see Fig. 3 for aligned sequences).

2.6. Generation of anti-T-cadherin peptide antibodies

A search for antigenic determinants of human T-cadherin was performed using the Peptide Companion sequence analysis program package (CohiSoft/PeptiSearch). The algorithm for predicting immunodominant regions is based on the hydrophilicity assessment of an amino acid sequence and on analysis of the occurrence probability of various amino acid combinations in the known antigenic determinants [25,26]. On the basis of these methods potential antigenic determinants were designated and peptides corresponding to the following amino acid positions of human T-cadherin precursor were synthesized: 140–160; 161–179; 260–271 (Fig. 3). The peptides were prepared by solid phase synthesis on Applied Biosystems 431A Synthesizer using Fmoc-amino acyl polymers (Bachem, Switzerland) and conjugated with keyhole limpet hemocyanin and bovine serum albumin. The content of peptides in conjugates was determined by quantitative amino acid analysis. Immunization of rabbits (three animals for each peptide) with the conjugates was performed according to standard protocol [20]. All experiments utilizing antibodies were performed using IgG fraction obtained by ammonium sulfate precipitation [20]. The concentration of immunoglobulins was determined by ELISA.

3. Results

We have developed an original procedure for purification of p105 from microsomes obtained from the media layers of human aortae. The procedure includes solubilization of proteins with CHAPS, three chromatographic (anion-exchange, metal-chelating and immunoaffinity) and two electrophoretic (one- and two-dimensional) steps (see Section 2). The course of purification is illustrated in Fig. 1 which presents characteristics for Coomassie staining (Panel A) and ligand (LDL) binding (Panel B) after the different steps of purification. Binding of LDL to the purified p105 was inhibited by pretreatment of the protein with 2-mercaptoethanol and by inclusion of EDTA into the incubation medium (Fig. 1C). These properties are indicative of specific mechanisms of p105-LDL interaction and are in accordance with our previous observations in nonfractionated membrane solubilizates of VSMCs [16].

Purity of the final preparation was checked by analytical 2-D gel electrophoresis. Fig. 2 presents Coomassie-stained gels (panels A, B) and corresponding ligand (LDL) blots (panels C, D) of starting membrane extract (panels A, C) and of the final p105 preparation (panels B, D) which was used for de-

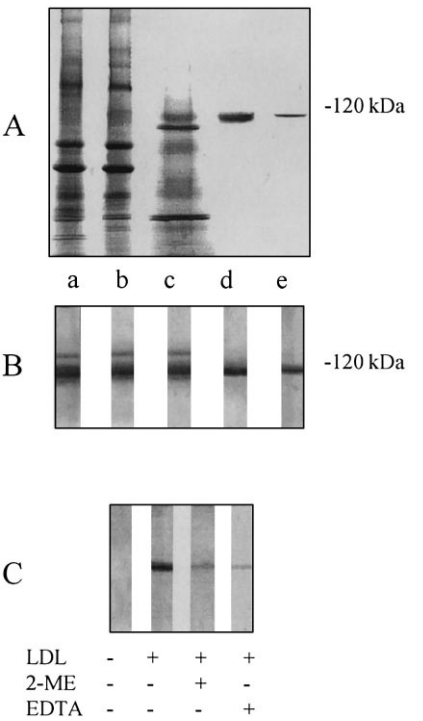

Fig. 1. The course of purification of p105 and LDL-binding characteristics. Coomassie-stained gels (panel A) and ligand blots (panel B) are presented to illustrate protein patterns and LDL-binding activity of p105 preparations at different stages of purification. The samples are: starting CHAPS extract (a) and preparations after DEAE (b) and metal-chelating chromatography (c), one- (d) and two-dimensional (e) gel electrophoresis. Panel C illustrates the ability of 2-mercaptoethanol and EDTA to inhibit binding of LDL to p105. 2-Mercaptoethanol was included in the electrophoresis sample buffer, while EDTA (20 mM) was added directly to the LDL incubation medium.

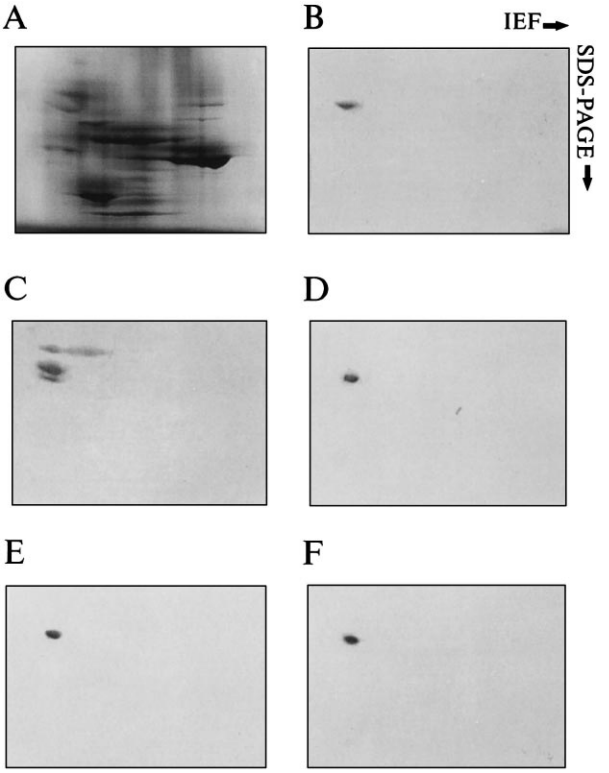

Fig. 2. Analysis of purity of the final p105 preparation by two-dimensional gel electrophoresis. Starting CHAPS extract and purified p105 were analysed either by Coomassie staining of 2-D gels (A, B) or by 2-D ligand blotting (C, D). Panels E and F represent 2-D Western blots of the final p105 preparation stained with 1 to 50 dilution of antisera to T-cadherin peptides 140–160 and 260–271, respectively. The left and right sides of the gel are the acidic (pH ≈ 4.5) and the basic (pH ≈ 8.5) sides, respectively.

termination of amino acid sequence. Note that the starting extract contained a major p105 spot and two minor spots of the same isoelectric point but slightly different molecular weights of ≈130 and 95 kDa, while the final preparation contained only p105. The nature of these additional LDL-binding proteins (which are also consistently observed in one-dimensional blots, see Fig. 1) and their relation to p105 is currently under investigation.

Purified p105 was further processed for determination of amino acid sequence. Three partial NH_2-terminal amino acid sequences of tryptic fragments from p105 were obtained. Comparative analysis of these sequences (SWISS-PROT database) revealed identity with the sequence of human T-cadherin [19] (Fig. 3).

On the basis of known full sequence of human T-cadherin, three potentially immunogenic peptides were selected for synthesis and used for immunization of rabbits (see Fig. 3 for positions of peptide residues). All three antibody preparations (but not control serum) recognized the same 130, 105 and 95 kDa bands on one-dimensional Western blots of aortic membrane extracts, and immunoreactivity colocalized with ligand (LDL) binding (data not shown). The identity of immuno- and ligand blotting patterns was also observed on two-dimensional Western blots as illustrated for two antisera in Fig. 2D, E and F. Nonimmune rabbit antiserum did not interact with p105 on 2-D blots (data not shown).

Similarly to classical cadherins [27], p105 was resistant to trypsin in the presence of Ca^{2+}, while in EDTA-containing medium the protein was rapidly degraded with simultaneous loss of LDL-binding activity (data not shown). Treatment of cultured human aortic smooth muscle cells with GPI-specific phospholipase C [18] resulted in a loss of at least 80% of cell-associated p105 and corresponding accumulation of soluble protein of the same electrophoretic mobility recognized by anti-peptide antibody (E. Kuzmenko, manuscript in preparation). These data demonstrate that p105 is expressed on cell surface and is anchored in membrane through a GPI group. Since T-cadherin is the only currently known GPI-anchored cadherin, these data support our identification of p105 as T-cadherin.

We tested antisera against synthetic T-cadherin peptides 140–160, 161–179 and 260–271 for their ability to interfere with LDL binding to p105. Inclusion of these antibodies during incubation of blots with LDLs dose dependently blocked binding of the lipoprotein (Fig. 4A and B). The most prominent inhibitory effect was exerted by antibodies directed to the peptide located near the NH_2-terminus of mature T-cadherin (starting from position 139 of the precursor), while the antiserum to the peptide positioned closer to C-terminus was the least efficient (Fig. 4A).

4. Discussion

We have previously demonstrated the existence of atypical lipoprotein-binding proteins (major p105, minor p130) in membranes of human and rat VSMCs [16]. Characterization of lipoprotein-binding properties revealed features of a ligand-receptor interaction rather than a mere nonspecific adhesion of lipoprotein to a sticky protein. Specifically, the binding is selective for LDLs, is divalent cation dependent, requires intact disulfide bonds in the lipoprotein-binding proteins and can be inhibited by anti-LDL (apo B) antibodies. Additionally, lipoprotein binding is not enhanced by high ionic

1 MQPRTPLVLC VLLSQVLLLT SAEDLDCTPG FQQKVFHINQ PAEFIEDQSI LNLTFSDCKG NDKLRYEVSS PYFKVNSDGG LVALRNITAV

91 GKTLFVHART PHAEDMAELV IVGGKDIQGS LQDIFKFART SPVPRQKRS*I VVSPILIPE*N QRQPFPRDVG K*VVDSDRPE*R SKFRLTGKGV

↑ (140-160) ↑ (161-179)

181 DQEPKGIFRI NENTGSVSVT RTLDREVIAV YQLFVETTDV NGKTLEGPVP LEVIVIDQND NRPIFREGPY IGHVMEGSPT GTTVMRMTAF

↑ (260-271)

271 DADDPATDNA LLR*YNIR*QQT PDKPSPNMFY IDPEKGDIVT VVSPALLDRE TLENPKYELI IEAQDMAGLD VGLTGTATAT IMIDDKNDHS

361 PKFTKKEFQA TVEEGAVGVI VNLTVEDKDD PTTGAWRAAY TIINGNPGQS FEIHTNPQTN EGMLSVVKPL DYEISAFHTL LIKVENEDPL

451 VPDVSYGPSS TATVHITVLD VNEGPVFYPD PMMVTRQEDL SVGSVLLTVN ATDPDSLQHQ TIRYSVYKDP AGWLNINPIN GTVDTTAVLD

541 RESPFVDNSV YTALFLAIDS GNPPATGTGT LLITLEDVND NAPFIYPTVA EVCDDAKNLS VVILGASDKD LHPNTDPFKF EIHKQAVPDK

631 VWKISKINNT HALVSLLQNL NKANYNLPIM VTDSGKPPMT NITDLRVQVC SCRNSKVDCN AAGALRFSLP SVLLLSLFSL ACL

Fig. 3. Complete amino acid sequence of human T-cadherin (as precursor form) obtained from the SWISS-PROT database. Italics indicate experimentally derived amino acid sequences of p105 proteolytic fragments. Processed protein contains residues 140 and further. Peptides selected for synthesis and subsequent use in raising antibodies are underlined. Arrows indicate the start of each peptide.

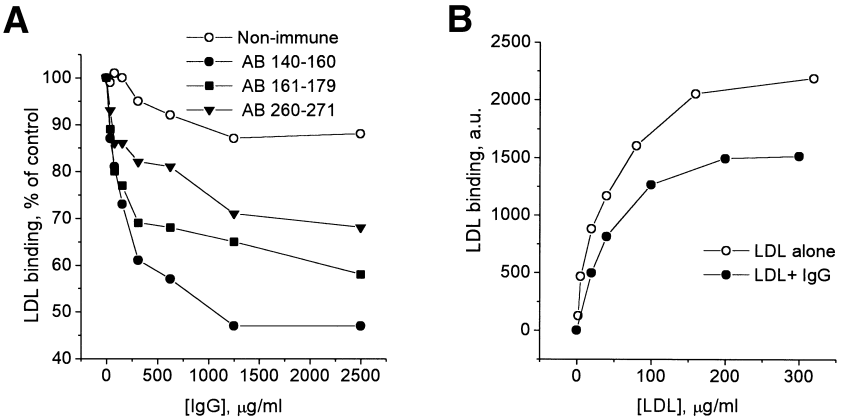

Fig. 4. Inhibition of LDL/p105 binding by anti-T-cadherin peptide antibodies. Equal amounts (50 μg) of p105 preparation obtained after metal (Zn^{2+}) chelate chromatography were electrophoresed, blotted onto nitrocellulose and incubated either with 40 μg/ml of LDL in the presence of increasing concentrations of total IgG (panel A), or with varying concentrations of LDL in the presence or absence of a fixed amount (2500 μg/ml) of immunoglobulins anti 140–160 (panel B). LDL binding was quantitated using peroxidase-conjugated anti-LDL. Comparable results with respect to both the degree of inhibition and relative efficiency of different antisera were obtained in two additional experiments.

strength favoring hydrophobic interactions and cannot be inhibited by polycations or polyanions [16,17]. The nature/identities of these lipoprotein-binding proteins was not known. In this present work we have purified p105 to homogeneity and presented evidence that p105 is identical with T-cadherin. This conclusion is based on the results of direct sequencing of purified p105 and the data demonstrating the ability of anti-peptide antibodies to T-cadherin to react with p105 on Western blots and to block LDL binding to p105 on ligand blots.

Cadherins are plasma membrane proteins mediating selective cell-cell recognition. They bind to other molecules identical to themselves situated on adjacent cells; this phenomenon is called homophilic binding (reviewed in [28]). Some characteristics of p105 (T-cadherin)/LDL binding correlate with known properties of these cell adhesion molecules. Firstly, binding mediated by cadherins requires divalent cations [29] and LDL binding to p105 (T-cadherin) is inhibited by EDTA (Fig. 1C; [16]). Secondly, cadherin-mediated cell-cell adhesion has been shown to be sensitive to dithiothreitol [29] and LDL binding to p105 (T-cadherin) is decreased after reduction of samples with 2-mercaptoethanol (Fig. 1C; [16]). Thirdly, the homotypic interaction site of cadherins is located close to the NH_2-terminus of the molecules and antibodies selectively blocking E-, P- or N-cadherin activity all recognize epitopes localized within about 30 residues from the NH_2-termini [30]; our results with antisera indicate that most prominent inhibition of LDL binding to p105 (T-cadherin) is exerted by the antibody recognizing a peptide positioned at the NH_2-terminus of mature T-cadherin which begins from the position 140 of the precursor (Fig. 4A). These similarities suggest that mechanisms of LDL/T-cadherin recognition may be analogous to those mediating homophilic cadherin/cadherin interactions.

The present data indicate that cadherins may have a less stringent ligand selectivity than was previously assumed. Our unequivocal identification of T-cadherin as a lipoprotein-binding protein shows that cadherins have the potential to interact with soluble ligands. Such heterophilic interactions may be relevant to alteration of cadherin-cadherin recognition and thereby the functions of T-cadherin in intercellular binding/communication. Further studies on the role of lipoproteins in regulation of cell-cell interactions and T-cadherin-mediated intracellular signalling are required to examine these issues.

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