

T-cadherin and signal-transducing molecules co-localize in caveolin-rich membrane domains of vascular smooth muscle cells

M.P. Philippova^a, V.N. Bochkov^{a,*}, D.V. Stambolsky^a, V.A. Tkachuk^a, T.J. Resink^b

^aLaboratory of Molecular Endocrinology, Institute of Experimental Cardiology, Cardiology Research Center, 121552 Moscow, Russia

^bCardiovascular Research Laboratories, Department of Research, Basel University Hospitals, 4031 Basel, Switzerland

Received 28 April 1998; revised version received 11 May 1998

Abstract Cadherins are a family of cellular adhesion proteins mediating homotypic cell-cell binding. In contrast to classical cadherins, T-cadherin does not possess the transmembrane and cytosolic domains known to be essential for tight mechanical coupling of cells, and is instead attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. This study explores the hypothesis that T-cadherin might function as a signal-transducing protein. Membranes from human and rat vascular smooth muscle cells were fractionated using Triton X-100 solubilization and density gradient centrifugation techniques. We demonstrate that T-cadherin is enriched in a minor detergent-insoluble low-density membrane domain and co-distributes with caveolin, a marker of caveolae. This domain was enriched in other GPI-anchored proteins (CD-59, uPA receptor) and signal-transducing molecules (G α s protein and Src-family kinases), but completely excluded cell-cell and cell-matrix adhesion molecules (N-cadherin and β 1-integrin). Coupling of T-cadherin with signalling molecules within caveolae might enable cellular signal transduction.

© 1998 Federation of European Biochemical Societies.

Key words: Lipoprotein; Cadherin; Vascular smooth muscle cell

1. Introduction

We have previously demonstrated the existence of atypical lipoprotein-binding proteins in vascular smooth muscle cells which may be involved in signalling effects of lipoproteins [1,2]. The ligand selectivity of these proteins of 105 and 130 kDa (p105/p130) is distinct from those of all other recognized lipoprotein receptor [3]. Purification and sequencing of the 105 kDa protein from human aortic media enabled identification of p105 as T-cadherin, an unusual member of the cadherin family of cell adhesion molecules [4]. Unlike the classical cadherin-family members T-cadherin does not have transmembrane and cytosolic domains and is anchored to membranes by means of a glycosylphosphatidylinositol (GPI) anchor [5]. The classical and desmosomal cadherins should be anchored to intracellular filaments in order to function properly [6,7]. In vivo and in vitro studies have shown that defects in structure or expression of either cytosolic cadherin domains or the proteins linking cadherins and desmogleins/desmocol-

lins to their corresponding types of filament result in impaired cell-cell adhesion, tissue disintegration and acquisition of invasive properties by cells [8–11]. Although T-cadherin has been demonstrated to mediate cell-cell adhesion in vitro, it was noted that in contrast to classical cadherins, T-cadherin was not concentrated to sites of cell-cell contact in monolayer cultures of transfected cells [12]. Further studies showed that in polarized epithelial cells T-cadherin localized on apical cell surfaces, while classical cadherins were present on the basolateral pole [13]. These data support the view that the lack of the cytosolic domain in T-cadherin might endow this molecule with intracellular targeting properties and specific function(s) that are distinct from classical and desmosomal cadherins.

A large number of GPI-anchored proteins have been implicated in signal transduction processes [14–20], and it therefore seems plausible that T-cadherin may be a signalling-coupled receptor. T-cadherin has been shown to negatively regulate axon growth in neurons, and to reduce neurite growth by repulsing growth cones rather than by arresting their growth through increased adhesion [21]. T-cadherin has also been shown to mediate contact growth inhibition in breast cancer cells [22]. These data suggest that in addition to some function in mechanical coupling of cells, T-cadherin may possess signalling functions and mediate intercellular communication.

The mechanisms of putative T-cadherin-mediated signalling events may be similar to those of other GPI-anchored proteins, some of which are known as signal-transducing receptors [14–20]. Signal transduction by GPI-anchored proteins has been hypothesized to involve their clustering in low-density, detergent-insoluble plasma membrane domains in which a variety of signalling proteins such as, among others, growth factor receptors, G-proteins, kinases of the Src family, and Ras proteins are co-localized [23–29]. In some cell types, including vascular smooth muscle cells, these detergent-resistant membrane domains are enriched with a specific protein, caveolin, and form morphologically conspicuous plasma membrane invaginations known as caveolae [30]. This study explores a possible function for T-cadherin as a signal-transducing molecule. We demonstrate a co-localization of T-cadherin, caveolin and some signal-transducing molecules in detergent-insoluble low-density membrane domains of vascular smooth muscle cells (VSMC).

2. Materials and methods

2.1. Materials

Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Delipidated dry milk, Rapilait, was from Migros (Basel, Switzerland). Cell culture reagents were from Gibco BRL (Life Technologies AG, Basel, Switzerland). Triton X-100, buffers and other chemicals were purchased from Sigma (St. Louis, MO, USA).

*Corresponding author. Fax: (7) (095) 414-67-19.

E-mail: bochkov@aha.ru

Abbreviations: VSMC, vascular smooth muscle cells; GPI, glycosylphosphatidylinositol; LDL, low-density lipoprotein; pAB, polyclonal antibody; mAB, monoclonal antibody; uPA, urokinase-type plasminogen activator

2.2. Isolation of detergent-insoluble, low-density membrane domains

Triton-insoluble, low-density membrane domains were purified from human or rat VSMC and human aortic media tissue by ultracentrifugation in stepwise sucrose density gradients as described previously [30]. VSMC were isolated, characterized and propagated as described [31]. VSMC were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics as described [31]. Confluent VSMC monolayers were rinsed with PBS and scraped into MES buffer saline (MBS, 25 mM 2-(*N*-morpholino)ethanol sulfonic acid, pH 6.5 at 4°C, 0.15 M NaCl, 2 µg/ml leupeptin, 2 µg/ml aprotinin and 1 mM PMSF) containing 1% Triton X-100. Membranes from human aortic media were isolated from thoracic aortae obtained within 5–8 h of accidental death as described before [1] and solubilized in MBS/Triton X-100. After homogenization with 10–15 strokes of a Dounce homogenizer extracts were adjusted to 40% sucrose (w/v) and placed in the bottom of centrifuge tubes. A stepwise gradient (5 and 30% sucrose in MBS without Triton X-100) was formed above the lysates and the tubes were centrifuged at 35 000 rpm for 18 h at 4°C in an SW 40 rotor (Beckman Instruments). A single turbid band was harvested from the interface between the 30 and 5% sucrose layers, diluted with 5 volumes of MBS without Triton X-100 and pelleted by centrifugation at 35 000 rpm for 2 h in an SW 40 rotor. The pellets were frozen in liquid nitrogen and stored at –70°C for later use. For ligand- and immunoblotting experiments Triton-insoluble pellets or starting membranes were solubilized by incubation for 2 h, at 4°C with Tris-buffered saline (50 mM Tris-HCl, pH 8.0 at 4°C, 150 mM NaCl, 2 µg/ml leupeptin, 2 µg/ml aprotinin and 1 mM PMSF) containing 1% SDS. In several experiments protein distribution over the whole sucrose gradient was studied. In these cases, sucrose gradients after ultracentrifugation (12 ml per tube) were fractionated into 1-ml fractions for rat samples or into 230-µl fractions for human samples and analyzed for turbidity (optical density at 560 nm), protein content (bicinchoninic acid assay, Pierce, Zurich) and subjected to immuno- and ligand-blotting.

2.3. Generation of anti-T-cadherin peptide antibody

Peptide corresponding to human T-cadherin precursor amino acid

positions 161–179 was prepared by solid phase synthesis on an Applied Biosystems 431A Synthesizer using Fmoc-amino acyl polymers (Bachem, Switzerland). Conjugation of peptide, immunization of animals, characterization of antiserum and purification of IgG fraction were performed as described previously [4,32].

2.4. Ligand- and immunoblotting

The method of immuno- and ligand-blotting with LDL has been fully described previously [1,3]. Briefly, samples were electrophoresed in 8% SDS polyacrylamide gels and electroblotted onto nitrocellulose membranes. A molecular mass standard mixture (Rainbow high range labelled markers, Amersham, Zurich, Switzerland, Cat# RPN 756) consisting of myocin (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa) was used for calibration. LDL for ligand blotting assays (1.019–1.063 g/ml) were isolated from the plasma of healthy male humans using sequential buoyant density centrifugation technique with the use of sodium bromide for density adjustments as described [33]. LDL was biotinylated using D-biotin-*N*-hydroxysuccinimide (0.15 µmol/mg LDL) [34]. LDL-binding protein bands were detected after sequential incubation of blots with biotinylated LDL, streptavidin-horseradish peroxidase conjugate and the Amersham ECL detection system, with final exposure to Kodak Biomax film. Immunoblotting procedures were essentially the same except that detection of bound primary antibodies was performed using anti-mouse, anti-rabbit or anti-goat secondary antibody conjugated to horseradish peroxidase (Transduction Laboratories, Zurich, Switzerland) and 0.1% Tween 20 was included in all incubating and washing solutions. The following commercially available primary antibodies were used for immunoblotting: anti-caveolin pAB (0.025 µg/ml, Transduction Lab., Cat# C13630), anti-clathrin heavy chain mAB (0.025 µg/ml, Transduction Lab., Cat# C43820), anti-integrin β1 mAB (0.025 µg/ml, Transduction Lab., Cat# I41720), anti-c-*Src* pAB (0.05 µg/ml, Santa Cruz Biotechnology, Cat# sc-18), anti-Gαs pAB (0.2 µg/ml, Santa Cruz Biotechnology, Cat# sc-823), anti-N-cadherin pAB (0.2 µg/ml, Santa Cruz Biotechnology, Cat# sc-1502), anti-urokinase receptor pAB (1 µg/ml, Amer-

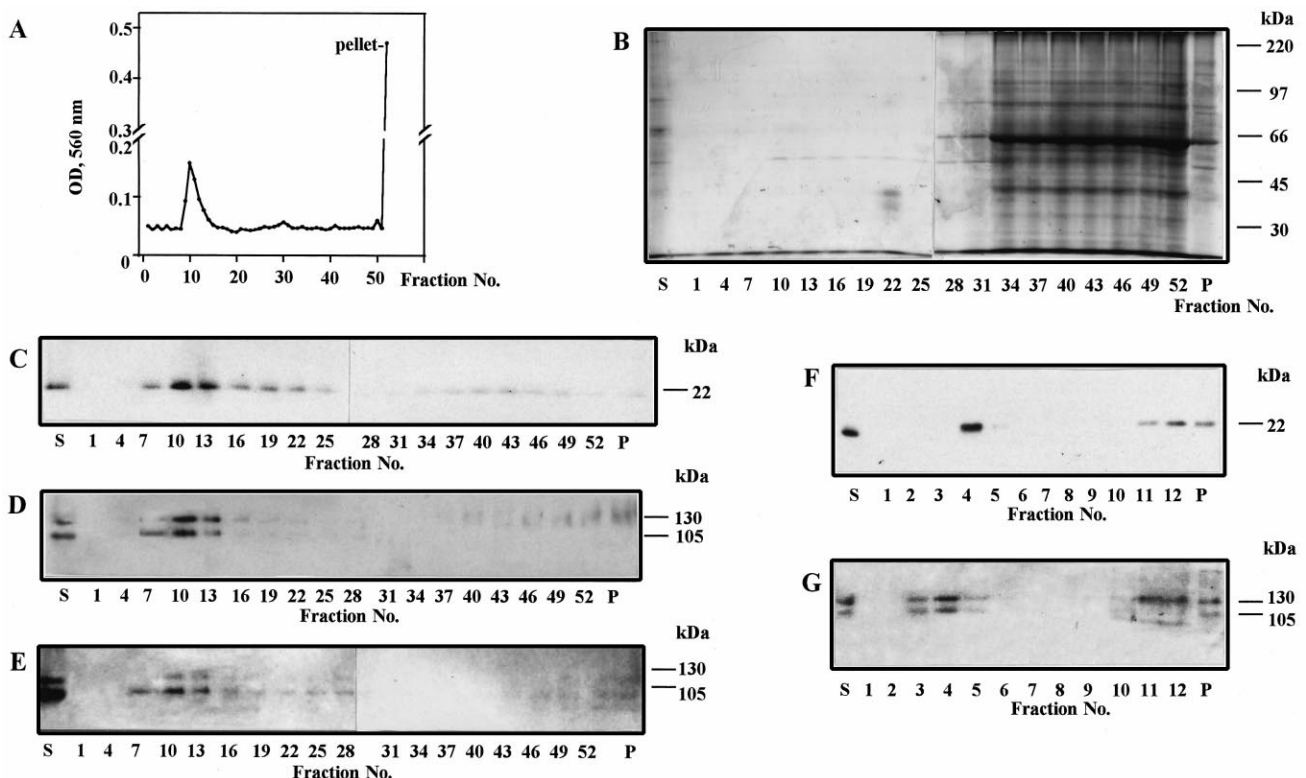


Fig. 1. Separation of Triton X-100 extract of VSMC by ultracentrifugation in a stepwise sucrose gradient: centrifugation profiles. Membranes of human aortic media and cultured rat VSMC were solubilized in MES-buffered solution containing 1% Triton X-100, adjusted to 40% sucrose, transferred to centrifuge tubes and overlaid with 30 and 5% sucrose solutions. After ultracentrifugation the gradients were fractionated and analyzed for turbidity, protein content and also subjected to immuno- and ligand-blotting. S = starting lysates; P = pellets. A: Optical density at 560 nm, human aorta; B: Coomassie R-250 staining, human aorta; C: anti-caveolin pAB, human aorta; D: anti-T-cadherin pAB, human aorta; E: LDL binding, human aorta; F: anti-caveolin pAB, rat VSMC; G: LDL binding, rat VSMC.

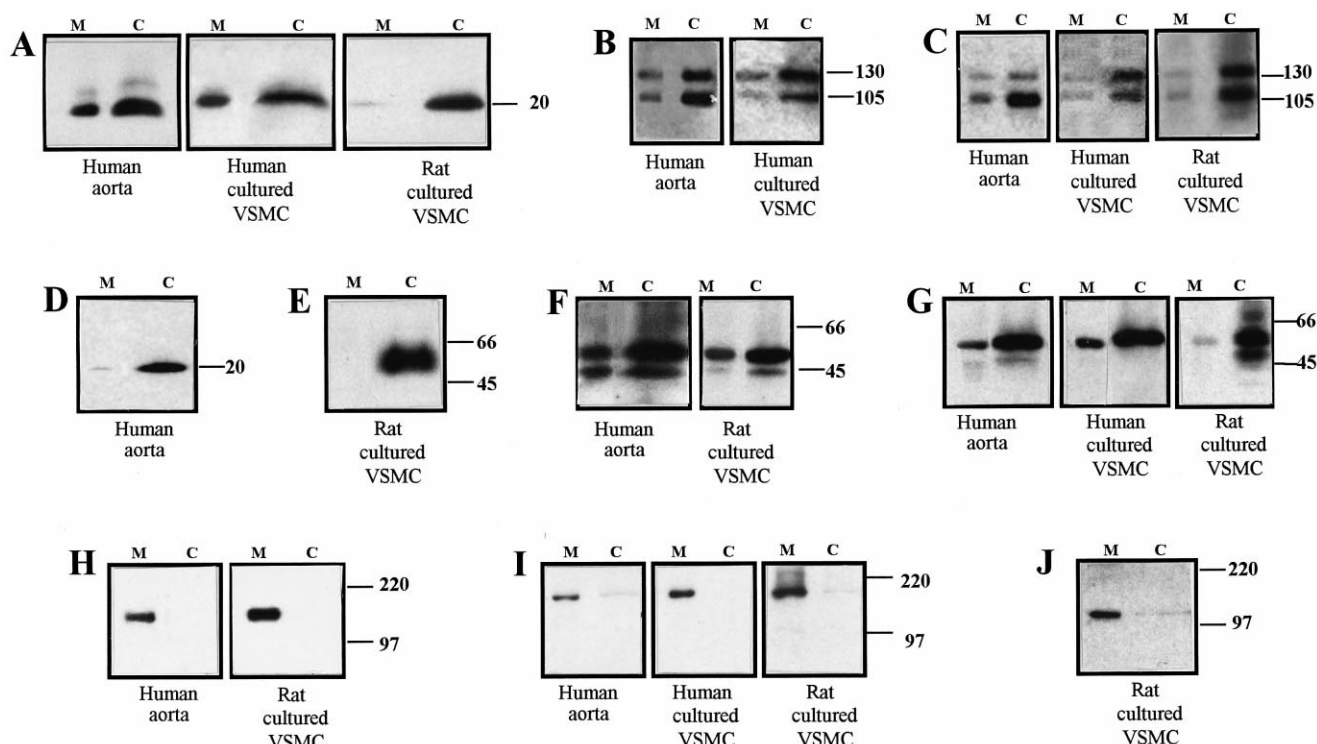


Fig. 2. Enrichment of T-cadherin and other lipid-anchored proteins in detergent-insoluble, low-density membrane domains of VSMC. Starting membrane (M) and 'caveolae' preparations (C) from human aortic media and cultures of human and rat VSMC were solubilized in Tris-buffered solution containing 1% SDS, loaded on 8% SDS-PAGE (15 µg of protein per lane), electroblotted onto nitrocellulose membrane and analyzed by ligand blotting with LDL (C) or by immunoblotting with anti-caveolin (A), anti-T-cadherin (B), anti-CD-59 (D), anti-uPA receptor (E), anti-Gαs (F), anti-c-Src kinases (G), anti-integrin β1 (H), anti-clathrin heavy chain (I) or anti-N-cadherin (J) antibodies. Numbers to the right of each panel indicate molecular weights.

ican Diagnostica, Cat# 3920). Anti-human CD59 mAb was a generous gift from Dr. A. Shamshiev, Laboratory for Experimental Immunology, Basel University Hospitals.

3. Results and discussion

3.1. Distribution of T-cadherin and other membrane-associated proteins in VSMC membrane fractions

In contrast to classical cadherins, desmosomal cadherins and protocadherins, T-cadherin is a GPI-anchored protein. A characteristic of GPI proteins is their enrichment in cholesterol and glycolipid-rich detergent-insoluble low-density membrane domains, which in most cell types form morphologically evident membrane invaginations known as caveolae [30]. In order to determine whether T-cadherin also localizes to the detergent-insoluble, low-density domains we applied a centrifugation-based technique widely used for isolation of caveolae [30]. After ultracentrifugation of Triton X-100-treated human aortic media membranes the low-density membrane domain insoluble in Triton X-100 was clearly visible as a turbid band at the interphase between 30% and 5% sucrose (Fig. 1A). Analysis of proteins in the centrifugation profile by SDS gel electrophoresis demonstrated that the bulk of protein was present in the lower, high-density sucrose fractions (Fig. 1B). A well-known marker for caveolae, the 21-kDa protein caveolin, was highly enriched in the low-density fractions corresponding to the turbid interphasic band (Fig. 1C). Immunoblotting with anti-T-cadherin pAB and LDL-blotting analysis revealed that T-cadherin (Fig. 1D) was co-distributed with caveolin (Fig. 1C). The LDL-binding protein p130 was also

recognised by anti-T-cadherin pAB and exhibited a distribution that was identical to the p105 LDL-binding and anti-T-cadherin immunoreactive protein (Fig. 1D,E). Some of our recent data (manuscript in preparation) would suggest that p130 is a partially processed form of T-cadherin precursor that is expressed on the cell surface simultaneously with the mature protein. Low-density membrane fractions from rat VSMC also displayed co-distribution of LDL-binding proteins p105 and p130 and caveolin (Fig. 1F,G).

3.2. Co-localization of T-cadherin and signalling molecules within caveolae

In order to compare enrichment of T-cadherin in low-density fractions with the distribution of other membrane-associated proteins, we performed ligand- and immunoblotting experiments on starting membranes and 'caveolae' fractions from human aortic media and cultures of human and rat VSMC. In each case low-density 'caveolae' fractions were enriched in both caveolin (Fig. 2A) and T-cadherin/LDL-binding (Fig. 2B,C). 'Caveolae' were significantly enriched with the GPI-anchored proteins CD-59 and uPA receptor (Fig. 2D,E) as well as with other lipid-anchored proteins, Gαs and Src-family kinases (Fig. 2F,G). In contrast, the typical transmembrane proteins β1-integrin and classical N-cadherin were excluded from the low-density 'caveolae' compartment (Fig. 2H,J). Clathrin, a membrane-associated protein involved in receptor-mediated endocytosis [35], was also excluded from 'caveolae' (Fig. 2I). These data support that T-cadherin, like some other GPI proteins, is directed by means of a lipid anchor to the detergent-insoluble,

cholesterol- and glycolipid-rich, low-density membrane domains.

3.3. T-cadherin as a signal-transducing protein

Cadherins are known to participate in cell-cell adhesion, establishment of cell polarity and morphogenesis in a variety of organs [36–41]. Emerging data suggest that their function is not limited to physical adhesion between cells, but may involve generation of intracellular signals [42,43]. While some data supporting an important role of signalling in cadherin function have been obtained [44,45], the mechanisms of signal transduction by cadherins are still obscure.

While possessing the general extracellular domain structure typical of classical type I cadherins, T-cadherin lacks the cytosolic domain and is anchored to the plasma membrane by a GPI anchor. The present study has demonstrated that a further peculiarity of T-cadherin is its localization to the detergent-resistant, low-density domains of plasma membrane. These results are in agreement with recent data demonstrating a similar distribution of T-cadherin in sheep ventricular sarcolemma [46]. Caveolae and caveolin-free low-density membrane lipid 'rafts' have been proposed to function as plasma membrane signal transduction centers that compartmentalize receptors with downstream effectors [23,30,47]. Our data show a distinct co-localization of T-cadherin and some recognized signal-transducing effectors such as non-receptor tyrosine kinases and G protein subunits within caveolae. Furthermore, we have shown that T-cadherin is located in membrane domains which exclude classical transmembrane cell adhesion molecules. Thus we may suppose that, rather than serving merely adhesive functions, T-cadherin may also function as a signal-transducing protein. The mechanisms whereby T-cadherin transduces the initial signal and the signalling effectors to which T-cadherin might couple are, however, completely unknown. At present we cannot assess whether T-cadherin may use signal transduction pathways similar to those utilized by other GPI proteins. Further investigations should be directed toward a search for signalling 'adaptor' molecules which may be either common or distinct between T-cadherin and other GPI-anchored proteins localized in caveolae.

Acknowledgements: This work was supported by grants from the Swiss National Foundation (31-41874.94), the Swiss Heart Foundation, the Swiss Academy of Medical Sciences, the Velux Stiftung, the Russian Foundation for Basic Research (95-04-12253) and INTAS (93-3260).

References

- [1] Kuzmenko, Y.S., Bochkov, V.N., Philippova, M.P., Tkachuk, V.A. and Resink, T.J. (1994) *Biochem. J.* 303, 281–287.
- [2] Bochkov, V.N., Tkachuk, V.A., Kuzmenko, Y.S., Borisova, Y.L., Buhler, F.R. and Resink, T.J. (1994) *Mol. Pharmacol.* 45, 262–270.
- [3] Bochkov, V.N., Tkachuk, V.A., Philippova, M.P., Stambolsky, D.V., Buhler, F.R. and Resink, T.J. (1996) *Biochem. J.* 317, 297–304.
- [4] Tkachuk, V.A., Bochkov, V.N., Philippova, M.P., Stambolsky, D.V., Kuzmenko, E.S., Sidorova, M.V., Molokoedov, A.S., Sprov, V.G. and Resink, T.J. (1998) *FEBS Lett.* 421, 208–212.
- [5] Ranscht, B. and Dours-Zimmermann, M.T. (1991) *Neuron* 7, 391–402.
- [6] Koch, P.J. and Franke, W.W. (1994) *Curr. Opin. Cell Biol.* 6, 682–687.
- [7] Green, K.J. and Jones, J.C.R. (1996) *FASEB J.* 10, 871–881.
- [8] Ozawa, M., Engel, J. and Kemler, R. (1990) *Cell* 63, 1033–1038.
- [9] Takeichi, M. (1993) *Curr. Opin. Cell Biol.* 5, 806–811.
- [10] Fujimori, T. and Takeichi, M. (1993) *Mol. Biol. Cell* 4, 37–47.
- [11] Okazaki, M., Takeshita, S., Kawai, S., Kikuno, R., Tsujimura, A., Kudo, A. and Amman, E. (1994) *J. Biol. Chem.* 269, 12092–12098.
- [12] Vestal, D.J. and Ranscht, B. (1992) *J. Cell Biol.* 119, 451–461.
- [13] Koller, E. and Ranscht, B. (1996) *J. Biol. Chem.* 271, 30061–30067.
- [14] Fischer, G.F., Majdic, O., Gadd, S. and Knapp, W. (1990) *J. Immunol.* 144, 638–641.
- [15] Lee, J.-D., Kravchenko, V., Kirkland, T.N., Han, J., Mackman, N., Moriarty, A., Leturq, D., Tobias, P.S. and Ulevitch, R.J. (1993) *Proc. Natl. Acad. Sci. USA* 90, 9930–9934.
- [16] Bohuslav, J., Horejsi, V., Hansmann, C., Stockl, J., Weidle, U.H., Majdic, O., Bartke, I., Knapp, W. and Stockinger, H. (1995) *J. Exp. Med.* 181, 1381–1390.
- [17] Jing, S., Wen, D., Yu, Y., Holst, P.L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R., Louis, J.-C., Hu, S. and Altrock, B.W. (1996) *Cell* 85, 1113–1124.
- [18] Mason, J.C., Yarwood, H., Tarnok, A., Sugars, K., Harrison, A.A., Robinson, P.J. and Haskard, D.O. (1996) *J. Immunol.* 157, 874–883.
- [19] Noda, S., Kosugi, A., Saitoh, S., Narumiya, S. and Hamaoka, T. (1996) *J. Exp. Med.* 183, 2355–2360.
- [20] Airas, L., Niemela, J., Salmi, M., Puurunen, T., Smith, D.J. and Jalkanen, S. (1997) *J. Cell Biol.* 136, 421–431.
- [21] Fredette, B.J., Miller, J. and Ranscht, B. (1996) *Development* 122, 3163–3171.
- [22] Lee, S.W. (1996) *Nature Med.* 2, 776–782.
- [23] Anderson, R.G.W. (1993) *Proc. Natl. Acad. Sci. USA* 90, 10909–10913.
- [24] Smart, E.J., Ying, Y.-S., Mineo, C. and Anderson, R.G.W. (1995) *Proc. Natl. Acad. Sci. USA* 92, 10104–10108.
- [25] Arni, S., Ilangumaran, S., van Echten-Deckert, G., Sandhoff, K., Poincellet, M., Briol, A., Rungger-Brandt, E. and Hoessli, D.C. (1996) *Biochem. Biophys. Res. Commun.* 225, 801–807.
- [26] Liu, P., Ying, Y., Ko, Y.-G. and Anderson, R.G.W. (1996) *J. Biol. Chem.* 271, 10299–10303.
- [27] Parolini, I., Sargiacomo, M., Lisanti, M.P. and Peschle, C. (1996) *Blood* 87, 3783–3794.
- [28] Solomon, K.R., Rudd, C.E. and Finberg, R.W. (1996) *Proc. Natl. Acad. Sci. USA* 93, 6053–6058.
- [29] Song, K.S., Li, S., Okamoto, T., Quilliam, L.A., Sargiacomo, M. and Lisanti, M.P. (1996) *J. Biol. Chem.* 271, 9690–9697.
- [30] Lisanti, M.P., Tang, Z., Scherer, P.E. and Sargiacomo, M. (1995) *Methods Enzymol.* 250, 655–668.
- [31] Scott-Burden, T., Resink, T.J., Hahn, A.W.A., Baur, U., Box, R.J. and Buhler, F.R. (1989) *J. Biol. Chem.* 264, 12582–12589.
- [32] Harlow, E. and Lane, D. (1988) *Antibodies. A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- [33] Havel, R.J., Eder, H.A. and Bragon, H.H. (1955) *J. Clin. Invest.* 34, 1345–1353.
- [34] Roach, P.D. and Noel, S.-P. (1987) *J. Lipid Res.* 28, 1508–1514.
- [35] Harrison, S.C. and Kirchhausen, T. (1983) *Cell* 33, 650–652.
- [36] Takeichi, M. (1990) *Annu. Rev. Biochem.* 59, 237–252.
- [37] Takeichi, M. (1991) *Science* 251, 1451–1455.
- [38] Geiger, B. and Ayalon, O. (1992) *Annu. Rev. Cell Biol.* 8, 307–332.
- [39] Kemler, R. (1992) *Semin. Cell Biol.* 3, 149–155.
- [40] Ranscht, B. (1994) *Curr. Opin. Cell Biol.* 6, 740–746.
- [41] Behrens, J. (1994) *Acta Anat.* 149, 165–169.
- [42] Trolice, M.P., Pappalardo, A. and Peluso, J.J. (1997) *Endocrinology* 138, 107–113.
- [43] Kinch, M.S., Petch, L., Zong, C. and Burridge, K. (1997) *Cell Adhes. Commun.* 4, 425–437.
- [44] Saffell, J.L., Walsh, F.S. and Doherty, P. (1992) *J. Cell Biol.* 118, 663–670.
- [45] Williams, E.J., Furness, J., Walsh, F.S. and Doherty, P. (1994) *Neuron* 13, 583–594.
- [46] Doyle, D.D., Goings, G.E., Upshaw-Earley, J., Page, E., Ranscht, B. and Palfrey, H.C. (1998) *J. Biol. Chem.* 273, 6937–6943.
- [47] Simons, K. and Ikonen, E. (1997) *Nature* 387, 569–572.