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Biochemical and Biophysical Research Communications 327 (2005) 112–116
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Ectodomain shedding of human Nogo-66 receptor homologue-1 by zinc metalloproteinases

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Received 22 November 2004
Available online 9 December 2004

Abstract

The Nogo-66 receptor (NgR) plays a pivotal role in the inhibition of neuroregeneration as the receptor for multiple neurite outgrowth inhibitors such as Nogo-A. We have previously shown that NgR undergoes zinc metalloproteinase-mediated ectodomain shedding in neuroblastoma cells. Here, we demonstrate that the NgR-related protein NgR homologue-1 is released from neuroblastoma cells as a full-length ectodomain (NgRH1-ecto) and an N-terminal fragment (NTF-NgRH1) containing the leucine-rich repeat region of the protein. Inhibitors of the major protease classes failed to block the release of NgRH1-ecto, suggesting that this occurs via a protease-independent mechanism, presumably by a phospholipase-like enzyme. The release of NTF-NgRH1 was blocked by a hydroxamate-based zinc metalloproteinase inhibitor and tissue inhibitor of metalloproteinases-2 and -3, but not -1, implicating the involvement of membrane-type matrix metalloproteinases in this process. Our findings thus highlight the parallels between the ectodomain shedding of NgRH1 and that previously described for NgR.

Keywords: Ectodomain shedding; Neurite outgrowth inhibition; Nogo-66 receptor homologue-1; Zinc metalloproteinase

Regeneration of neurons in the central nervous system (CNS) following injury is severely compromised by the neurite outgrowth inhibitory activity of myelin-associated proteins such as Nogo-A [1–3], myelin-associated glycoprotein (MAG) [4], oligodendrocyte-myelin glycoprotein (OMgp) [5,6], and the chondroitin sulphate proteoglycans brevican and versican V2 [7,8]. The C-terminal extracellular domain of Nogo-A, Nogo-66, binds to the Nogo-66 receptor (NgR), a neuronal glycosylphosphatidylinositol (GPI)-anchored protein that forms a signal transduction complex with the transmembrane proteins $p75^{NTR}$ and LINGO-1 [9–12]. Even though they are structurally unrelated to Nogo-66, MAG and OMgp are also functional ligands for NgR [5,13,14]. The ligand binding domain of NgR is located within the N-terminal region of the receptor and contains eight leucine-rich repeats (LRRs) flanked by N- and C-terminal sub-domains (LRRNT and LRRCT, respectively) [15,16].

Database searches have recently identified two proteins with high sequence homology to NgR that have been termed Nogo-66 receptor homologues-1 and -2 (NgRH1 and NgRH2, respectively) [15–17]. Indeed, akin to NgR, NgRH1 and NgRH2 are predominantly expressed within the CNS, are anchored to the cell surface via a GPI moiety, and contain an LRRNT/LRR/LRRCT domain. However, despite these similarities, NgRH1 and NgRH2 fail to bind to Nogo-66, MAG, and OMgp and, consequently, their functions within the CNS remain to be elucidated [16].

We have recently demonstrated that human NgR expressed in human neuroblastoma SH-SY5Y cells is constitutively cleaved by zinc metalloproteinase(s) to liberate a soluble N-terminal fragment containing the entire ligand binding domain of the receptor and a lipid raft-associated C-terminal fragment [18]. In the present

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.12.001

study, we have addressed the question whether NgRH1 and NgRH2 undergo ectodomain shedding similar to NgR.

Materials and methods

Antibodies and inhibitors. The anti-V5 antibody, anti-FLAG antibody, and anti-HA peroxidase-conjugated antibody were obtained from Invitrogen, Stratagene, and Roche, respectively. The anti-NgRH1 rabbit polyclonal was raised against synthetic peptides corresponding to residues 350–365 and 366–380 of human NgRH1 as described previously [17]. Recombinant human tissue inhibitors of metalloproteinases were obtained from R&D Systems.

Cell lysis and medium preparation. SH-SY5Y cells stably expressing either human NgR with an N-terminal V5 tag, human NgRH1 with an N-terminal FLAG tag or human NgRH2 with an N-terminal HA tag have been described previously [17,18]. Following incubation in Opti-MEM, cells were washed twice with phosphate-buffered saline (PBS, Invitrogen), scraped into PBS, and harvested by centrifugation at 100*g* for 3 min. The cell pellet was resuspended in M-PER (Pierce) containing protease inhibitors (Roche) and incubated at 4 °C for 30 min prior to clarification by centrifugation at 13,000*g* for 5 min. Conditioned medium was clarified by centrifugation at 100*g* for 3 min. Digestion with peptide *N*-glycosidase F (PNGase F) was performed according to the manufacturer's instructions (Glyko).

SDS–PAGE and Western blot analysis. SDS–gel electrophoresis was performed using NuPAGE 4–12% bis-Tris gels (Invitrogen). Proteins were transferred to polyvinylidene difluoride membranes (Amersham) which were blocked by incubation for 1 h with Tris-buffered saline/0.1% Tween 20 (TBST) containing 5% dried milk powder followed by an incubation for 1 h in the same buffer containing 200 ng/ml anti-V5 antibody, 2 mg/ml anti-FLAG antibody peroxidase-conjugated antibody or a 1000-fold dilution of the anti-NgRH1 polyclonal antibody. Membranes were washed in TBST and incubated with the appropriate peroxidase-conjugated secondary antibody. Signals were developed using ECL Western Blotting Detection Reagents (Amersham) and Hyperfilm ECL (Amersham) according to the manufacturer's instructions.

Results

To investigate whether NgRH1 and NgRH2, like NgR, were subjected to ectodomain shedding in neuronal cells, SH-SY5Y cells stably expressing human NgR, NgRH1 or NgRH2 were incubated for 24 h with Opti-MEM (Fig. 1). As reported previously [18], NgR was detected by the anti-V5 antibody as a band of around 64 kDa in the cell lysate corresponding to the full-length receptor and a 48 kDa band in both the lysate and the incubation medium corresponding to the soluble N-terminal fragment of NgR. NgRH1 was detected by the anti-FLAG antibody as a band of around 55 kDa in the cell lysate and two bands of around 55 and 46 kDa in the medium. As the FLAG tag was on the N-terminus of NgRH1, the bands in the medium can only correspond to N-terminal fragments of the protein. NgRH2 was detected by the anti-HA antibody as bands of around 60 and 48 kDa in the cell lysate and the medium. However, the intensity of the bands in the medium was barely above the level of detection, indicating that NgRH2, unlike NgR and NgRH1, was not subjected to significant ectodomain shedding. To characterise the N-terminal fragments of NgRH1 shed into the medium, cell lysate and medium samples were de-glycosylated with PNGase F (Fig. 2B). Following deglycosylation, the anti-FLAG antibody detected a band of 42 kDa in the lysate corresponding to un-glycosylated full-length NgRH1; and bands of 42 and 31 kDa in the medium (termed A and B, respectively), most likely corresponding to the un-glycosylated counterparts of the 55 and 46 kDa bands observed in Fig. 1, respectively. As band A in the medium was also recognised by an anti-NgRH1 polyclonal antibody raised against peptides encompassing residues 350–380 of NgRH1 (Figs. 2A and B), this fragment extends at least beyond residue 350. Indeed, as the predicted molecular weight of the NgRH1 ectodomain is 42 kDa, band A most likely represents this domain (termed NgRH1-ecto). That band B was not detected by the anti-NgRH1 antibody demonstrates that the C-terminus of this N-terminal fragment (termed NTF-NgRH1) occurs at least before residue 380. As the molecular weight of un-glycosylated NTF-NgRH1 is higher than that predicted for the N-terminal region of NgRH1 terminating at the end of the LRRCT (28 kDa), NTF-NgRH1 must contain an intact LRRNT/LRR/LRRCT domain. Although the C-terminal fragment arising from the cleavage that generated NTF-NgRH1 should have been recognised in the cell lysate by the anti-NgRH1 polyclonal antibody, no band was detected other than that for the un-glycosylated full-length protein. As the GPI-anchored prion protein (PrP) has been shown to be released from cells as a full-length protein attached to membrane vesicles known as exosomes [19], we next addressed whether the 55 kDa band in the medium corresponded to full-length NgRH1 present on such vesicles (Fig. 2C). However, that the 55 kDa band, like the 46 kDa band, was only detected in the supernatant following ultracentrifugation of the medium demonstrates that it was not membrane-associated NgRH1 but rather a soluble fragment of the protein.

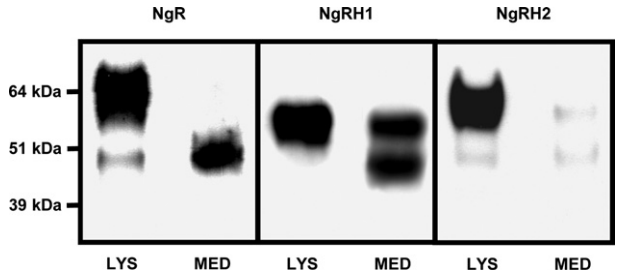

Fig. 1. Ectodomain shedding of NgR and NgRH1. SH-SY5Y cells expressing human NgR, NgRH1 or NgRH2 were incubated for 24 h with Opti-MEM and the proteins were detected in the lysate and medium by Western blot analysis with the anti-V5, anti-FLAG, and anti-HA antibodies, respectively.

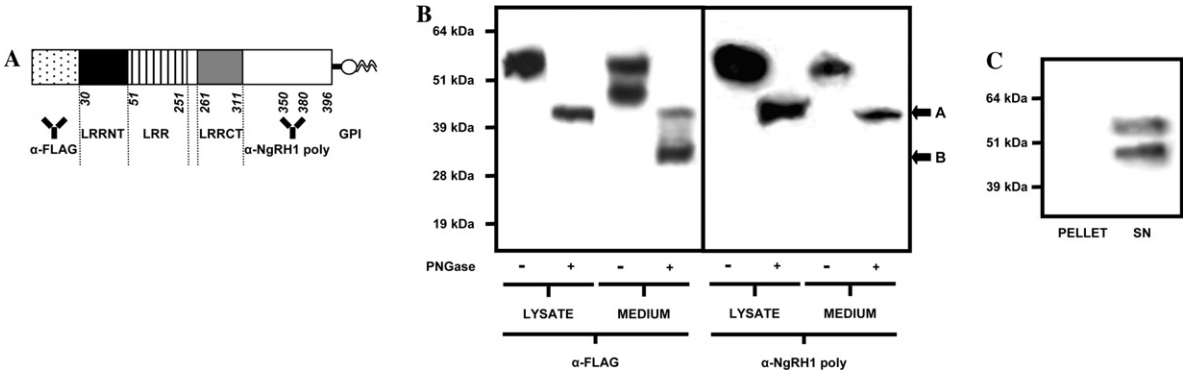

Fig. 2. Characterisation of the released NgRH1 fragments. (A) Schematic of the human NgRH1 construct used in the present study showing the N-terminal FLAG tag (dotted box), the LRRNT (black), LRR (lined box), and LRRCT (grey) sub-domains. Amino acid positions (italics) correspond to those for non-tagged human NgRH1. The region recognised by the anti-NgRH1 polyclonal antibody (α-hNgRH1 poly) and the C-terminal GPI-anchor (GPI) are indicated. (B) SH-SY5Y cells expressing NgRH1 were incubated for 24 h with Opti-MEM, and lysate and medium samples were incubated with (+) or without (−) PNGase F. NgRH1 was detected by Western blot analysis with the anti-FLAG or anti-NgRH1 polyclonal antibodies. Deglycosylated fragments corresponding to NgRH1-ecto and NTF-NgRH1 are indicated as (A,B), respectively. (C) Medium from a 24 h incubation with cells expressing NgRH1 was centrifuged at 100,000*g* for 1 h and NgRH1 was detected in the pellet and supernatant (SN) by the anti-FLAG antibody.

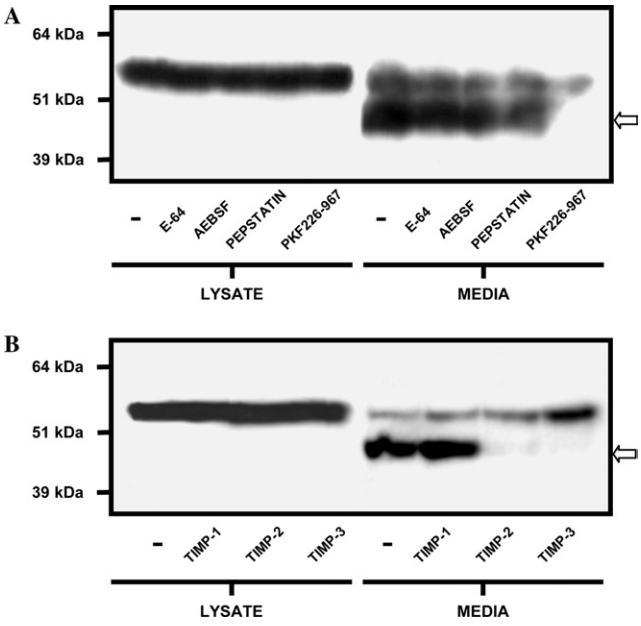

Fig. 3. Effect of protease inhibitors on NgRH1 shedding. (A) SH-SY5Y cells expressing NgRH1 were incubated for 24 h with Opti-MEM in the absence (−) or presence of either 10 μM E-64, 10 μM AEBSF, 10 μM pepstatin or 10 μM PKF226-967. NgRH1 in the lysate and medium was detected by Western blot analysis with the anti-FLAG antibody. NTF-NgRH1 is indicated with an open arrow. (B) Cells were incubated for 24 h with Opti-MEM in the absence (−) or presence of 10 μg/ml of the indicated recombinant human TIMP. NgRH1 in the lysate and medium was detected by Western blot analysis with the anti-FLAG antibody. NTF-NgRH1 is indicated with an open arrow.

To elucidate the proteases involved in the shedding of NgRH1, cells were treated with inhibitors of the major protease classes (Fig. 3A). Inhibitors of cysteine proteases (E-64), serine proteases (AEBSF) or aspartic proteases (pepstatin) had no effect on the shedding of NgRH1. On the other hand, PKF226-967, a broad spectrum hydroxamate-based inhibitor of zinc metalloproteinases [18], blocked the release of NTF-NgRH1 into the medium, but had no effect on the release of NgRH1-ecto. In order to further specify the type of zinc metalloproteinase involved in the release of NTF-NgRH1, cells were treated with tissue inhibitors of metalloproteinases (TIMPs) (Fig. 3B). TIMP-2 and TIMP-3 inhibit all matrix metalloproteinases (MMPs) and, in the case of TIMP-3, some ADAM (a disintegrin and metalloproteinase) members, whereas TIMP-1 inhibits ADAM10 and MMPs with the exception of most membrane-type (MT) MMPs [20]. TIMP-2 and TIMP-3, but not TIMP-1, blocked the release into the medium of NTF-NgRH1, suggesting that the release of this fragment was mediated by a member of the MT-MMP family. However, as seen for PKF226-967, none of the TIMPs had any effect on the release of NgRH1-ecto.

Discussion

We have previously reported the mutual biochemical properties of NgR, NgRH1, and NgRH2 in terms of their GPI-anchorage and lipid raft localisation on the cell surface [17]. We now demonstrate that human NgRH1 is subjected to ectodomain shedding in human neuroblastoma cells similar to NgR. The negligible amount of shedding of NgRH2 cannot be attributed to any defects in the trafficking of this protein as NgRH2, like NgR and NgRH1, has been shown to be localised on the surface of SH-SY5Y cells [17]. The shedding of NgRH1 exhibits several differences to that described for NgR [18]. First, NgRH1 is released as a full-length ectodomain and an N-terminal fragment,

whereas NgR is released as a single N-terminal fragment (termed NTF-NgR). Second, NTF-NgR can be detected intracellularly whereas NgRH1-ecto and NTF-NgRH1 were found only in the medium. Finally, the C-terminal fragment arising from the cleavage of NgR is readily detectable in cell lysates but no such fragment could be detected for NgRH1 using the anti-NgRH1 polyclonal antibody. Despite these differences, the release of NTF-NgR and NTF-NgRH1 from SH-SY5Y cells was inhibited by PKF226-967, TIMP-2, and TIMP-3; but not TIMP-1, indicating that NTF-NgR and NTF-NgRH1 are both generated by the action of a member of the MT-MMP group of zinc metalloproteinases [18,20]. That NTF-NgRH1 does not extend beyond residue 380 and, from its molecular weight, must terminate after the LRRNT/LRR/LRRCT domain indicates that the cleavage generating this fragment occurs within the region 312–379. Accordingly, NTF-NgR has been shown to terminate at residue 358 which, by sequence alignment, corresponds to residue 341 in NgRH1 [17]. Although there is low homology between NgR and NgRH1 around this site (SAGNA↓LKGRV and EAGAP↓PADPS, respectively), it must be noted that MT-MMPs have no known consensus sequence for substrate cleavage.

Unlike NTF-NgRH1, the release of NgRH1-ecto was insensitive not only to PKF226-967, but also to inhibitors of other major protease classes. As the size of NgRH1-ecto suggests that it comprises the full-length ectodomain of NgRH1, it is conceivable that it is released by cleavage within the GPI anchor of the protein by a phospholipase-like enzyme. Indeed, the mechanism by which the full-length ectodomains of NgR and the GPI-anchored prion protein (PrP) are released from SH-SY5Y cells has been postulated to involve a phospholipase-like enzyme based on the insensitivity of their release to inhibitors of zinc metalloproteinases [18,21]. However, whereas the release of NgRH1-ecto is constitutive, the release of the ectodomains of NgR and PrP can only be instigated by cholesterol depletion of the cells [18,21]. The ectodomains of other GPI anchored proteins such as the urokinase receptor, decay-accelerating factor, and basic fibroblast growth factor-binding heparan sulfate proteoglycan have been shown to be constitutively released from cells by the action of endogenous GPI-specific phospholipase D [22–24]. Whether this phospholipase is responsible for the release of NgRH1-ecto remains to be established.

Although NgRH1 fails to bind NgR ligands [16], the fact that it contains an LRRNT/LRR/LRRCT domain indicates that there is a high likelihood that it is the receptor of an as yet unidentified ligand(s). If this were indeed the case, it can be envisaged that ectodomain shedding would play a key role in modulating the function of NgRH1. For example, as NgRH1-ecto and NTF-NgRH1 contain the entire LRRNT/LRR/LRRCT domain of the protein, they may be proficient in ligand binding, but as they lack a membrane-anchoring domain, they may be incapable of signaling and thus act as ligand antagonists. This would parallel the scenario for the shedding of NgR, where NTF-NgR binds Nogo-66 but fails to associate with the NgR co-receptor $p75^{NTR}$ and, as such, may act as an antagonist of NgR ligands [18].

References

[1] M.S. Chen, A.B. Huber, M.E. van der Haar, M. Frank, L. Schnell, A.A. Spillmann, F. Christ, M.E. Schwab, Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1, Nature 403 (2000) 434–439.

[2] T. GrandPre, F. Nakamura, T. Vartanian, S.M. Strittmatter, Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein, Nature 403 (2000) 439–444.

[3] R. Prinjha, S.E. Moore, M. Vinson, S. Blake, R. Morrow, G. Christie, D. Michalovich, D.L. Simmons, F.S. Walsh, Inhibitor of neurite outgrowth in humans, Nature 403 (2000) 383–384.

[4] L. McKerracher, S. David, D.L. Jackson, V. Kottis, R.J. Dunn, P.E. Braun, Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth, Neuron 13 (1994) 805–811.

[5] K.C. Wang, V. Koprivica, J.A. Kim, R. Sivasankaran, Y. Guo, R.L. Neve, Z. He, Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth, Nature 417 (2002) 941–944.

[6] V. Kottis, P. Thibault, D. Mikol, Z.C. Xiao, R. Zhang, P. Dergham, P.E. Braun, Oligodendrocyte-myelin glycoprotein (OMgp) is an inhibitor of neurite outgrowth, J. Neurochem. 82 (2002) 1566–1569.

[7] B.P. Niederöst, D.R. Zimmermann, M.E. Schwab, C.E. Bandtlow, Bovine CNS myelin contains neurite growth-inhibitory activity associated with chondroitin sulfate proteoglycans, J. Neurosci. 19 (1999) 8979–8989.

[8] M. Schmalfeldt, C.E. Bandtlow, M.T. Dours-Zimmermann, K.H. Winterhalter, D.R. Zimmermann, Brain derived versican V2 is a potent inhibitor of axonal growth, J. Cell Sci. 113 (2000) 807–816.

[9] A.E. Fournier, T. GrandPre, S.M. Strittmatter, Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration, Nature 409 (2001) 341–346.

[10] K.C. Wang, J.A. Kim, R. Sivasankaran, R. Segal, Z. He, p75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp, Nature 420 (2002) 74–78.

[11] S.T. Wong, J.R. Henley, K.C. Kanning, K.H. Huang, M. Bothwell, M.M. Poo, A p75(NTR) and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein, Nat. Neurosci. 5 (2002) 1302–1308.

[12] S. Mi, X. Lee, Z. Shao, G. Thill, B. Ji, J. Relton, M. Levesque, N. Allaire, S. Perrin, B. Sands, T. Crowell, R.L. Cate, J.M. McCoy, R.B. Pepinsky, LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex, Nat. Neurosci. 7 (2004) 221–228.

[13] B.P. Liu, A. Fournier, T. GrandPre, S.M. Strittmatter, Myelin-associated glycoprotein as a functional ligand for the Nogo-66 receptor, Science 16 (2002) 1190–1193.

[14] M. Domeniconi, Z. Cao, T. Spencer, R. Sivasankaran, K.C. Wang, E. Nikulina, N. Kimura, H. Cai, K. Deng, Y. Gao, Z. He, M. Filbin, Myelin-associated glycoprotein interacts with the

Nogo66 receptor to inhibit neurite outgrowth, Neuron 35 (2002) 283–290.
[15] X.L. He, F.J. Bazan, G. McDermott, J.B. Park, K. Wang, M. Tessier-Lavigne, Z. He, C. Garcia, Structure of the Nogo receptor ectodomain: a recognition module implicated in myelin inhibition, Neuron 38 (2003) 177–185.
[16] W.A. Barton, B.T. Liu, D. Tzvetkova, P.D. Jeffrey, A.E. Fournier, D. Sah, R. Cate, S. Strittmatter, D.B. Nikolov, Structure and outgrowth inhibitor binding of the Nogo-66 receptor and related proteins, EMBO J. 22 (2003) 3291–3302.
[17] V. Pignot, A.E. Hein, C. Barske, C. Wiessner, A.R. Walmsley, K. Kaupmann, H. Mayeur, B. Sommer, A.K. Mir, S. Frentzel, Characterization of two novel proteins, NgRH1 and NgRH2, structurally and biochemically homologous to the Nogo-66 receptor, J. Neurochem. 85 (2003) 717–728.
[18] A.R. Walmsley, G. McCombie, U. Neumann, D. Marcellin, R. Hillenbrand, A.K. Mir, S. Frentzel, Zinc metalloproteinase-mediated cleavage of the human Nogo-66 receptor, J. Cell Sci. 117 (2004) 4591–4602.
[19] B. Fevrier, D. Vilette, F. Archer, D. Loew, W. Faigle, M. Vidal, H. Laude, G. Raposo, Cells release prions in association with exosomes, Proc. Natl. Acad. Sci. USA 101 (2004) 9683–9688.
[20] A.H. Baker, D.R. Edwards, G. Murphy, Metalloproteinase inhibitors: biological actions and therapeutic opportunities, J. Cell Sci. 115 (2002) 3719–3727.
[21] E.T. Parkin, N.T. Watt, A.J. Turner, N.M. Hooper, Dual mechanisms for shedding of the cellular prion protein, J. Biol. Chem. 279 (2004) 11170–11178.
[22] C.N. Metz, G. Brunner, N.H. Choi-Muira, H. Nguyen, J. Gabrilove, I.W. Caras, N. Altszuler, D.B. Rifkin, E.L. Wilson, M.A. Davitz, Release of GPI-anchored membrane proteins by a cell-associated GPI-specific phospholipase D, EMBO J. 13 (1994) 1741–1751.
[23] G. Brunner, C.N. Metz, H. Nguyen, J. Gabrilove, S.R. Patel, M.A. Davitz, D.B. Rifkin, E.L. Wilson, An endogenous glycosylphosphatidylinositol-specific phospholipase D releases basic fibroblast growth factor–heparan sulfate proteoglycan complexes from human bone marrow cultures, Blood 83 (1994) 2115–2125.
[24] O.G. Wilhelm, S. Wilhelm, G.M. Escott, V. Lutz, V. Magdolen, M. Schmitt, D.B. Rifkin, E.L. Wilson, H. Graeff, G. Brunner, Cellular glycosylphosphatidylinositol-specific phospholipase D regulates urokinase receptor shedding and cell surface expression, J. Cell. Physiol. 180 (1999) 225–235.