Tumour necrosis factor α -converting enzyme mediates ectodomain shedding of Vps10p-domain receptor family members

Guido HERMEY¹, Susanne S. SJØGAARD, Claus Munck PETERSEN, Anders NYKJÆR and Jørgen GLIEMANN²

Institute of Medical Biochemistry, MIND Center, Ole Worms Allé, Building 1170, University of Aarhus, 8000 Århus C, Denmark

Several transmembrane molecules are cleaved at juxtamembrane extracellular sites leading to shedding of ectodomains. We analysed shedding of members of the Vps10p-D (Vps10p domain; where Vps is vacuolar protein sorting) family of neuronal type-I receptors with partially overlapping functions, and additional proteolytic events initiated by the shedding. When transfected into CHO (Chinese-hamster ovary) cells (CHO-K1), sorCS1a–sorCS1c isoforms were shed at high rates ($\sim 0.61\% \cdot \text{min}^{-1}$) that were increased approx. 3-fold upon stimulation with phorbol ester. sorCS1c identified in the cultured neuroblastoma cell line SH-SY5Y was shed similarly. In CHO-K1 transfectants, constitutive and stimulated shedding of sorCS3 also occurred at high rates (0.29% and $1.03\% \cdot \text{min}^{-1}$). By comparison, constitutive and stimulated shedding of sorLA occurred at somewhat lower rates (0.07% and $0.48\% \cdot \text{min}^{-1}$), whereas sorCS2 and sortilin were shed at very low rates even when stimulated ($\sim 0.01\% \cdot \text{min}^{-1}$). Except for sorCS2, shedding of the receptors was dramatically

reduced in mutant CHO cells (CHO-M2) devoid of active TACE (tumour necrosis factor α -converting enzyme), demonstrating that this enzyme accounts for most sheddase activity. The release of sorCS1 and sorLA ectodomains initiated rapid cleavage of the membrane-tethered C-terminal stubs that accumulated only in the presence of γ -secretase inhibitors. Purified shed sorLA bound several ligands similarly to the entire luminal domain of the receptor, including PDGF-BB (platelet-derived growth factor-BB) and amyloid- β precursor protein. In addition, PDGF-BB also bound to the luminal domains of sorCS1 and sorCS3. The results suggest that ectodomains shed from a subset of Vps10p-D receptors can function as carrier proteins.

Key words: Chinese hamster ovary (CHO) cell, ectodomain shedding, sorLA, sortilin, tumour necrosis factor α -converting enzyme, Vps10p-domain receptor.

INTRODUCTION

Protein ectodomain shedding is an important post-translational mechanism that regulates function of cell-surface proteins, including several transmembrane type-I receptors expressed in the central nervous system (for reviews, see [1–4]). Notch and p75^{NTR} (p75 neurotrophin receptor) are examples that illustrate shedding, leading to proteolysis of the membrane-tethered stub in a process called regulated intramembrane proteolysis (for a review, see [5]). After binding of ligand, Notch is cleaved at a membrane-proximal site by a sheddase/secretase identified as a member of the ADAM (a disintegrin and metalloproteinase) family, presumably ADAM17/TACE (tumour necrosis factor α -converting enzyme) [6]. The cleavage elicits subsequent γ -secretase-mediated proteolysis within the transmembrane segment of the stub [7] followed by release of the cd (cytoplasmic domain), which travels to the nucleus and participates in transcriptional activation of target genes [8]. On the other hand, p75^{NTR} is shed independently of binding of extracellular ligands such as the proNGF [proform of NGF (nerve growth factor)] [9]. The shedding is stimulated by activators of protein kinase C such as phorbol esters, and TACE was recently identified as the enzyme primarily responsible for the cleavage of p75^{NTR} in CHO (Chinese-hamster ovary) cells [10]. Ectodomain shedding of p75^{NTR} is a prerequisite for subsequent γ -secretase proteolysis and release of the p75^{NTR}-cd, which may participate in signalling events before its degrad-

ation in proteasomes [9,11]. In contrast with the full-length receptor and the stub generated by TACE-mediated shedding, the free p75^{NTR}-cd generated by γ -secretase activity is unable to associate with Trk tyrosine kinase receptors, and cleavage by α - and γ -secretases is therefore likely to regulate co-receptor functions of p75^{NTR} [11].

The family of multifunctional neuronal receptors containing an N-terminal Vps10p-D (Vps10p domain; where Vps is vacuolar protein sorting) comprises sortilin, whose ectodomain consists of the Vps10p-D only, sorLA/LR11, which has several additional domains in the luminal part, and sorCS1–3, which constitute a subgroup of mutually highly similar proteins containing both a Vps10p-D and a leucine-rich domain ([12] and references therein). The family members are type-I transmembrane proteins with short cds, and the mature receptors are generated after furin-mediated removal of N-terminal propeptides in the Golgi compartment. In addition, alternative splicing of the sorCS1 gene results in expression of at least three different isoforms (sorCS1a–sorCS1c) with completely different cds, of which the sorCS1c-cd is similar to the cds of sorCS2 and sorCS3, and the different cds mediate different trafficking of the receptor in cells [13,14]. Some Vps10p-D family members have specific functions not shared by others. For example, the ectodomain of sorLA exhibits avid binding of Alzheimer's disease-associated APP (amyloid precursor protein), and sorLA can protect the precursor from conversion into the toxic amyloid- β [15]. On

Abbreviations used: ADAM, a disintegrin and metalloproteinase; APP, amyloid precursor protein; cd, cytoplasmic domain; CHO, Chinese-hamster ovary; DAPT, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; GST, glutathione S-transferase; lactacystin, clasto-lactacystin β -lactone; NGF, nerve growth factor; p75^{NTR}, p75 neurotrophin receptor; PDGF-BB, platelet-derived growth factor-BB; proNGF, proform of NGF; RAP, receptor-associated protein; TACE, tumour necrosis factor α -converting enzyme; Vps, vacuolar protein sorting; Vps10p-D, Vps10p domain; wt, wild-type.

¹ Present address: Institute of Molecular Neurobiology, Free University of Berlin, Takustrasse 6, 14195 Berlin, Germany.

² To whom correspondence should be addressed (email jgliemann@biokemi.au.dk).

the other hand, several ligands are shared among the Vps10p-D receptors, suggesting overlapping functions. Thus proNGF, which binds to sortilin and creates a cell death signalling complex via simultaneous binding to p75^{NTR} [16], also binds to sorCS3 [17]. In addition, sortilin and sorLA both bind the neuropeptide neurotensin, the propeptides of sortilin, sorLA and sorCS1, components of the plasminogen-activating system, as well as the ER (endoplasmic reticulum) resident RAP (receptor-associated protein) [13,18–21].

The purpose of the present study was to analyse ectodomain shedding of Vps10p-D receptors and to identify the enzyme(s) involved. We show that whereas sorCS2 and sortilin transfected into CHO-K1 cells are shed at very low rates, constitutive and phorbol ester-stimulated shedding of sorCS1 and sorCS3 occur at high rates, and shedding of sorLA at intermediate rates. The sheddase activity is mainly accounted for by TACE, the extracellular cleavage initiates rapid cleavage of the membrane-tethered stubs, and shed ectodomain is capable of ligand binding.

MATERIALS AND METHODS

Reagents, antibodies and soluble receptor species

The metalloproteinase inhibitor GM6001 (galarin), PMA, lactacystin (clasto-lactacystin β -lactone) and neurotensin were obtained from Sigma–Aldrich. DAPT [*N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester; γ -secretase inhibitor IX], epoxomicin, MG132 and apolipoprotein E3 were from Calbiochem, and PDGF-BB (platelet-derived growth factor-BB) was from R & D Systems. APP fragment (amino acids 304–612) was from Sigma–Aldrich. APP₆₉₅ (the isoform with 695 amino acids) was kindly provided by Dr Roberto Cappai (Department of Pathology, University of Melbourne, Melbourne, Victoria, Australia) [15]. Recombinant RAP and NGF-prodomain–GST (glutathione *S*-transferase) fusion protein were prepared as described in [17,19]. Rabbit antibodies directed against the mature luminal part of human sorCS1 [amino acids 78–1067; anti-(L-sorCS1)], against its leucine-rich domain [amino acids 942–1023; anti-(leu-sorCS1)], against luminal sorCS3, luminal sortilin and the sortilin-cd, and luminal sorLA and the sorLA-cd, have been described [12,13,19,20,22]. Antisera against the cds of sorCS1a–sorCS1c were raised in rabbits using a GST fusion protein of the entire cd of human sorCS1a and synthetic peptides corresponding to distal parts of the mouse sorCS1b and sorCS1c cds (YRLQRPRPATPPSPKR and YRRPGQLVDEKVESQLLGK). Antisera raised against these peptides also recognized the human b- and c-tails. Antiserum against the luminal part of sorCS2 was raised in rabbits. The Ig fractions were purified from rabbit sera using Protein A–Sepharose and stored at concentrations of approx. 10 mg/ml. Soluble sorLA and His₆-tagged sorCS1 and sorCS3, secreted from cells transfected with cDNA covering the entire luminal domains, were purified as described in [13,17,20]. Shed sorLA was purified by RAP–Sepharose affinity chromatography similar to soluble sorLA [20], except that the medium was from incubates of cells expressing the full-length receptor with PMA (100 ng/ml) added 1 h before recovery.

Expression of receptor constructs and cell culture

CHO-K1 cells stably transfected with human sorCS1a–sorCS1c, sorCS3, and sortilin and sorLA have been described in [13,17,19,20]. For generation of ‘tailless’ sorCS1 (sorCS1-delta-cd), a 3′ fragment corresponding to the C-terminus of the luminal and transmembrane parts of sorCS1 (bp 2954–3360) was amplified by PCR using the primers 5′-CAAACCTGGATGACTA-

CAACCCCG and 5′-CTAGTAGATGACGAACACTGCCAGC. The fragment was cloned into the previously described pcDNA3.1/Zeo(+)-sorCS1 expression vector [13] via the native BamHI site (bp 3128) and the NotI site in the polylinker region of this vector, and construct for generation of tailless sortilin was produced similarly. Constructs for generation of human sorCS2, which is mainly expressed on the surface of CHO cell transfectants, will be described elsewhere (S. S. Sjøgaard and A. Nykjær, unpublished work).

CHO-K1 cells were transfected using FuGENE 6 (Roche Molecular Biochemicals) and stable transfectants were selected in a medium containing 300 μ g/ml Zeocin (Invitrogen). The mutated CHO subline M2 was kindly provided by Dr Joaquín Arribas (University Hospital Vall d'Hebron, Barcelona, Spain) and transfected as described for CHO-K1 cells. CHO-M2 cells produce two TACE variants with distinct point mutations (C225Y and C600Y), which are both catalytically inactive and also cause reduced exit from the ER, whereas the cells process wt (wild-type) TACE and other Zn-dependent metalloproteinases normally [23–25]. All transfected CHO cell lines were cultured in HyQ-CCM5 (Hyclone, Logan, UT, U.S.A.), containing 300 μ g/ml Zeocin and, for M2 cells, 500 μ g/ml G418. The human neuroblastoma cell line SH-SY5Y (ATCC number: CRL-2266) was cultured in DMEM (Dulbecco's modified Eagle's medium)/Ham's F-12 medium (1:1, v/v) supplemented with 10% (v/v) foetal bovine serum.

Incubations, Western blotting, metabolic labelling and immunoprecipitation

Cells were grown to approx. 80% confluence in 1.9 mm² wells ($\sim 5 \times 10^4$ cells/well). To measure shedding, cells were incubated in DMEM culture medium (300 μ l) at 37°C with or without additions and, unless otherwise stated, medium was recovered after 1 h followed by lysis of the cells in 100 μ l of lysis buffer (20 mM Tris/HCl, 10 mM EDTA, 150 mM NaCl and 1% Triton X-100, pH 8.0) containing proteinase inhibitors, including Complete Mini (Roche Molecular Biochemicals) as described in [19]. Samples were then subjected to reducing SDS/PAGE using 4–16% or 8–16% acrylamide gels, and in some cases PhastGel high-density gels (Amersham Biosciences) to identify low-molecular-mass bands. Following Western blotting, the content of receptor (shed or present in cells) was quantified using a Fujifilm LAS-1000 luminescence image analyser. Unless otherwise stated, the sample sizes were 28 μ l of medium and 2.5 μ l of lysate, i.e. the ratio of medium to lysate was 3.7 (28 μ l from 300 μ l of medium/2.5 μ l from 100 μ l of lysate). When appropriate, the media were concentrated 6-fold by using Centricon-30 (Millipore).

In some cases, cells were biolabelled using 400 μ Ci/ml ³⁵S-labelled cysteine and methionine (Pro-mix; Amersham Biosciences) followed by immunoprecipitation of receptor from total media and lysates using the appropriate antibody and GammaBind beads (Amersham Biosciences) followed by PAGE and autoradiography. Each experiment was performed at least three times, and the results were based on two different clones of the CHO-K1 and CHO-M2 transfectants. For preparation of CHO cell membranes, cells were washed and homogenized at 4°C in 10 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA and 250 mM sucrose supplemented with Complete Mini using a 22-gauge needle. Debris and nuclei were removed by centrifugation at 10 000 *g* for 10 min followed by centrifugation of the supernatant at 100 000 *g* for 45 min.

Immunocytochemistry

The cells were washed in PBS, fixed with 4% (w/v) paraformaldehyde in the same buffer, permeabilized using 0.5%

saponin (Sigma–Aldrich) followed by incubation with primary and secondary antibodies.

Surface plasmon resonance analyses

The analyses were performed on a BIAcore 3000 instrument equipped with CM5 sensor chips as described in [13,21] with receptor species immobilized to densities of approx. 50 fmol/mm². The overall K_d (dissociation constant) values were determined by BIAevaluation 3.0 software using a Langmuir 1:1 binding model.

RESULTS

Shedding of sorCS1 isoforms

Figure 1(A) shows absence of sorCS1 in lysate and medium of wt CHO-K1 cells incubated for 1 h as judged by Western blotting using the α -L-sorCS1 antibody. Similar experiments demonstrated little or no expression of other Vps10p-D receptors (results not shown). In sorCS1a transfectants, the 1 h medium shows an immunoreactive band migrating slightly faster than the full-length receptor present in the lysate, and immunoprecipitation using the anti-(leu-sorCS1) antibody confirmed the identity as sorCS1 ectodomain (results not shown). PMA (100 ng/ml) stimulated the shedding, in agreement with the observation that phorbol esters up-regulate activities of several sheddases [1–4], and half-maximal effect was obtained with 10–20 ng/ml PMA (results not shown). We used the wide-range hydroxamate-based inhibitor GM6001 (30 μ M) to determine if the shedding might involve a Zn-dependent metalloproteinase and, as shown in Figure 1(A), this inhibitor nearly blocked the constitutive shedding (half-maximal effect at $\sim 7 \mu$ M; results not shown) and partially inhibited shedding in the presence of PMA.

Other experiments showed the same pattern when the 1 h incubation at 37°C was performed in CHO culture medium or DMEM, and no shedding was observed at 4°C. A 15–60 min time course at 37°C showed progressively increasing ectodomain in the medium, and degradation of shed receptor was minimal, as no change in immunoreactivity was detected after re-incubation for 120 min in medium from 24 h incubates of wt CHO-K1 cells (results not shown).

Figure 1(B) shows shedding of sorCS1a–sorCS1c by CHO-K1 transfectants, and constitutive shedding was calculated to be in the order of 40% $\cdot$ h⁻¹. GM6001 inhibited constitutive shedding by 90% or more and caused a concomitant increase in full-length receptors in lysates, whereas inhibition was only partial in the presence of PMA. Thus the pattern of shedding is similar for the a–c isoforms, even though their expression on the cell surface is different at steady state (~ 10 , 45 and 30% respectively [13]). We also tested shedding of mutated sorCS1 expressed only on the cell surface due to deletion of the cd. As compared with the a–c isoforms, shedding of sorCS1-delta-cd was slightly lower in magnitude, and in this case GM6001 inhibited almost completely also in the presence of PMA, suggesting that stimulated cleavage of sorCS1a–sorCS1c in CHO cells occurs partially in cellular compartments inaccessible to the inhibitor.

To exclude a possible effect of PMA on receptor synthesis in the 1 h incubation period, shedding was also assessed in metabolically labelled transfectants, as exemplified by sorCS1c. Figure 1(C) shows that shedding of the labelled receptor after a 1 h chase in the absence and presence of GM6001 or PMA was similar to the results obtained by Western blotting. When the chase period was extended to 4 h, most of the labelled receptor had been released from the cells even in the absence of PMA (results not shown).

Figure 1 Shedding of sorCS1 in CHO cell transfectants

(A) wt CHO-K1 cells and CHO-K1-sorCS1a transfectants were grown in CHO culture medium (HyQ-CCM5) to approx. 80% confluence, washed, and incubated for 1 h in 300 μ l of the same medium followed by recovery of the medium and lysis of the cells in 100 μ l of lysis buffer. Samples were subjected to reducing SDS/PAGE and Western blotting using the anti-(L-sorCS1) antibody. Left, lysate (L) and medium (M) of wt CHO-K1 cells. Right, lysates and media from CHO-K1 transfectants with additions as indicated (100 ng/ml PMA; 30 μ M GM6001). (B) sorCS1a–sorCS1c and sorCS1-delta-cd transfectants were incubated in DMEM for 1 h without or with additions as indicated, followed by Western blotting. All sample sizes were 28 μ l for media and 2.5 μ l for lysates, providing a medium/lysate ratio of 3.7. (C) sorCS1c transfectants were biolabelled for 4.5 h in cysteine- and methionine-free medium, washed, and incubated for 1 h in full medium with and without GM6001 or PMA. The receptor was then immunoprecipitated from total media and lysates using the anti-(L-sorCS1) antibody, and subjected to reducing SDS/PAGE and autoradiography.

These results are similar to those reported previously for shedding of p75^{NTR} in transfected CHO-K1 cells [10].

We then searched for a cell type with endogenous expression of sorCS1, and initial experiments showed that in the human neuroblastoma cell line SH-SY5Y, both α -L-sorCS1 and anti-(leu-sorCS1) antibodies recognized a protein with a size compatible with sorCS1 (Figures 2A and 2B). Immunoprecipitation from lysates of SY5Y cells using both antibodies (Figures 2A and

Figure 2 Expression and shedding of sorCS1c in SH-SY5Y neuroblastoma cells

(A–C) Western blotting (left lanes) and immunoprecipitation (IP) followed by Western blotting (right lanes) of sorCS1c in SY5Y cell lysates using antibodies as indicated. (D and E) Immunocytochemistry of sorCS1c in SY5Y cells using primary antibodies as indicated and Alexa 488-conjugated anti-rabbit antibodies. (F and G) The cells were grown to near-confluence and incubated in serum-free medium for 1 h with additions as indicated (50 ng/ml PDGF-BB), followed by Western blotting with the anti-(L-sorCS1) antibody. The sample sizes were 28 µl of 6-fold concentrated media (M) and 28 µl of cell lysates (L), providing a medium/lysate ratio of 2.0.

2B) and reaction with antibody specific for the sorCS1c-cd (Figure 2C) showed that the detected protein was sorCS1c. The result was confirmed by immunocytochemistry using anti-(L-sorCS1) (Figure 2D), anti-(leu-sorCS1) (results not shown) and anti-(sorCS1c-cd) (Figure 2E) antibodies, and no reaction was observed on Western blots or by immunocytochemistry with antibody against the sorCS1a-cd and sorCS1b-cd (results not shown). Figure 2(F) shows pronounced PMA-stimulated shedding of sorCS1c in SY5Y cells that was inhibited by GM6001, whereas constitutive shedding was minor. Because PMA is an artificial stimulator, we also analysed the effect of PDGF-BB, which caused a moderate stimulation (Figure 2G), in agreement with the previously described effect on metalloproteinase-mediated shedding of growth-hormone receptor [26], and similar results were obtained when using CHO-K1-sorCS1c transfectants (results not shown).

We speculated that TACE might be involved in the shedding event, as reported for several other transmembrane molecules [2–4]. SorCS1a–sorCS1c and sorCS1-delta-cd were therefore transfected into the mutant CHO cell subline M2 devoid of catalytically active TACE. Figure 3 shows absence of shed

Figure 3 Reduced shedding of sorCS1 species in TACE-deficient CHO-M2 cells

CHO-M2 and CHO-K1 cell transfectants were incubated in parallel with or without PMA for 1 h, and media and lysates were analysed by Western blotting using the anti-(L-sorCS1) antibody. The medium/lysate ratio was 3.7 except for M2 cells transfected with sorCS1a; here the medium was concentrated 6-fold to give a medium/lysate ratio of 22.2.

receptor in 6-fold concentrated medium of the sorCS1a-M2 cell transfectants in contrast with the medium (not concentrated) of the control CHO-K1 transfectants. From assessment of the detection level by serial dilutions (results not shown), we calculated that shedding was reduced by 98% or more in the sorCS1a-M2 transfectants. In sorCS1b, sorCS1c and sorCS1-delta-cd M2 cell transfectants, shedding was reduced by approx. 90%, but not abolished, indicating that a minor part of the shedding may be accounted for by proteinases other than TACE. Although this difference remains unexplained, it can be concluded that TACE is the proteinase responsible for most shedding of sorCS1 isoforms in CHO-K1 cells.

Fate of the sorCS1 stub

We focused on sorCS1c whose cd of 59 amino acids is similar to those of sorCS3 and sorCS2. Figure 4(A) shows that in the absence of inhibitors, the anti-sorCS1c-cd antibody did not detect low-molecular-mass components in lysates of CHO-K1 transfectants (or SY5Y cells; results not shown) incubated in the absence or presence of PMA, demonstrating rapid clearance of the stub generated by TACE-mediated shedding. However, cd-containing component(s) accumulated in cultures supplemented for 16 h with the γ -secretase inhibitor DAPT (Figure 4A), which had no effect on the ectodomain shedding (results not shown). Additional experiments demonstrated that the cd-containing component is in the membrane pellet of the cells, and Figure 4(B) shows a size of approx. 8 kDa as estimated for the transmembrane domain plus the cd. As expected, generation of the stub was inhibited by GM6001 in CHO-K1 transfectants, particularly in the absence of PMA, and was below detection limit in M2 cell transfectants. It should be noted that accumulation of the 8 kDa stub was moderate, suggesting some degradation even in the presence of DAPT. Also, PMA had no marked effect, possibly because it promotes not only formation, but also degradation of the stub.

Because no low-molecular-mass components accumulated in cells in the absence of DAPT, the soluble cd released after cleavage of the stub must also be rapidly degraded. To analyse if the cd accumulates in the presence of proteasome inhibitors, incubations were performed in the presence of epoxomicin and lactacystin. These inhibitors proved toxic to the cells in long-term incubations

Figure 4 Identification of the sorCS1c stub

(A) CHO-K1-sorCS1c transfectants were incubated for 16 h in CHO culture medium with or without DAPT (10 μ M) and, when indicated, PMA was added 1 h before lysis of the cells followed by Western blotting using the anti-(sorCS1c-cd) antibody. (B) CHO-K1- and CHO-M2-sorCS1c transfectants were incubated for 1 h with additions as indicated followed by Western blotting of lysates using PhastGel to resolve low-molecular-mass components. (C) CHO-K1 transfectants were incubated for 1 h with DAPT (10 μ M), epoxomicin (5 μ M; EPO) or lactacystin (10 μ M; Lact) followed by lysis and immunoprecipitation using the anti-(sorCS1c-cd) antibody and Western blotting. The lysates were prepared from incubations in 60 mm dishes to provide heavy overloading of the gel with regard to full-length receptor.

(results not shown), and we therefore used incubation for 1 h followed by immunoprecipitation from lysates of large pools of cells to increase the intensity of the signal. Figure 4(C) confirms accumulation of the stub in the presence of DAPT, whereas little product accumulated in the presence of epoxomicin and lactacystin, and nothing < 8 kDa was detected. The proteasome inhibitor MG132 caused accumulation of the 8 kDa product, presumably because it also inhibits γ -secretase activity [9], but again

Figure 5 Shedding of sorCS3

(A) CHO-K1-sorCS3 transfectants were incubated for 1 h with additions as indicated, and media and lysates were analysed by Western blotting using antibody against the luminal part of sorCS3 (medium/lysate ratio 3.7). (B) CHO-M2 and CHO-K1 transfectants were incubated in parallel and media and lysates were analysed. The medium/lysate ratio was 3.7 for CHO-K1 and 22.2 for M2 cell transfectants.

no product compatible with the soluble sorCS1c-cd was observed (results not shown).

Additional experiments showed accumulation of sorCS1b and sorCS1a stubs only in the presence of DAPT, and the putative cds (49 and 41 amino acids respectively) remained undetectable even in the presence of proteasome inhibitors. We conclude that sorCS1 stubs are degraded rapidly, and that the a–c cds are unlikely to accumulate in cells.

Shedding of sorCS3

SorCS3 exhibits 69 % overall amino acid identity with sorCS1c. Figure 5(A) shows that its shedding is similar to that of sorCS1 species, and that GM6001 is an effective inhibitor even in the presence of PMA. In this regard, sorCS3 resembles the cell-surface-expressed sorCS1-delta-cd (cf. Figure 1B) in agreement with the observation that sorCS3 is mainly expressed on the cell surface [17]. The established ligand NGF-propeptide (200 nM) had no effect on shedding of sorCS3 (results not shown). Figure 5(B) shows that shedding of sorCS3 in M2 cell transfectants is reduced by > 90 % as compared with CHO-K1 transfectants, demonstrating that TACE is important in the shedding event.

Minor shedding of sorCS2

SorCS2 is also a homologue of sorCS1c (48 % overall amino acid identity), and the receptor is mainly expressed on the cell surface. However, the pattern of sorCS2 shedding is completely different from those of sorCS3 and sorCS1 species. Figure 6(A) shows a minor release of ectodomain to the medium of CHO-K1 transfectants in the absence of any additions, a minimal effect of PMA, and lack of inhibition by GM6001. Moreover, a minor shedding was also observed in M2 cell transfectants (Figure 6B). When taken together, the results suggest that TACE has only a minor role, if any, in the slow shedding of sorCS2.

Minor shedding of sortilin

The medium of CHO-K1 transfectants did not contain sufficient shed sortilin by 1 h to allow direct detection. A minor shedding that was stimulated by PMA (Figure 7A) and inhibited by

Figure 6 Minor shedding of sorCS2

(A) CHO-K1-sorCS2 transfectants were incubated for 1 h with additions as indicated followed by Western-blot analysis (medium/lysate ratio 3.7). (B) CHO-M2 and CHO-K1 transfectants were incubated in parallel and analysed (medium/lysate ratio 3.7).

Figure 7 Minor shedding of sortilin

(A) CHO-M2 and CHO-K1 cells transfected with full-length sortilin were incubated with additions as indicated followed by Western-blot analysis using a medium/lysate ratio of 22.2. (B) CHO-K1-sortilin-delta-cd transfectants were analysed using a medium/lysate ratio of 22.2.

GM6001 (results not shown) was detected in 6-fold concentrated medium. The established ligands NGF-propeptide (200 nM) [16], neurotensin (1 μ M), sortilin propeptide (0.5 μ M) and RAP (1 μ M) [19] had little or no effect (results not shown). The minor shedding in CHO-K1 transfectants was further reduced in M2 cell transfectants, suggesting that TACE is involved. Moreover, a product compatible with a sortilin stub appeared in CHO-K1 transfectants after overnight incubation with DAPT (results not shown). Because sortilin is 90–95% intracellular and might in part be inaccessible to TACE, we also analysed sortilin devoid of the cd and, as shown in Figure 7(B), the cell surface-expressed sortilin-delta-cd was also shed at very low rates. We conclude that shedding of sortilin is much slower than that of sorCS1 species and sorCS3. Although it follows that sortilin is a poor substrate for TACE, this convertase is likely to account for most of the observed shedding.

Shedding of sorLA

Figure 8(A) shows that sorLA, as compared with sorCS1 and sorCS3, is shed constitutively at a relatively low rate in CHO-K1 cells, in agreement with previous observations [21], and that PMA causes a marked stimulation. Other experiments showed that PDGF-BB caused a moderate increase in shedding, whereas the ligands RAP, neurotensin and sorLA propeptide [20] had little or no effect in the absence or presence of PMA (results not shown). Figure 8(B) shows that shedding is not detectable in M2 cell transfectants, demonstrating that TACE mediates the proteolytic

Figure 8 Shedding of sorLA

(A) CHO-K1-sorLA transfectants were incubated for 1 h with additions as indicated, and media and lysates were analysed by Western blotting using the anti-(L-sorLA) antibody. The medium/lysate ratio was 9.2 in the displayed example. (B) Media and lysates of CHO-M2 and CHO-K1 cell transfectants were analysed [anti-(L-sorLA) antibody] using a medium/lysate ratio of 22.2. (C) CHO-K1-sorLA transfectants were incubated for 16 h with or without DAPT and, when indicated, PMA was added for 1 h; lysates were analysed using the anti-(sorLA-cd) antibody. (D) wt CHO-K1 cells and CHO-K1-sorLA transfectants were incubated for 1 h as indicated followed by Western blotting [anti-(sorLA-cd) antibody] using PhastGel to resolve low-molecular-mass components.

cleavage. As seen in Figure 8(C), antibody directed against sorLA-cd detected minor amounts of low-molecular-mass component(s) in the absence of any additions, and DAPT caused a marked accumulation, suggesting that the sorLA stub is more resistant to cleavage than that of sorCS1c (cf. Figure 4A). Figure 8(D) shows that the low-molecular-mass component is approx. 8 kDa, as expected for a stub containing the 54-amino-acid sorLA-cd, and that its generation is inhibited by GM6001. Experiments were also performed in the presence of the proteasome inhibitors epoxomicin and lactacystin, but we were unable to detect any product < 8 kDa, suggesting rapid degradation of the sorLA-cd after cleavage of the stub (results not shown).

Comparison of shedding rates

After stimulation with PMA, the cells were no longer at steady state, and shedding of e.g. the 'fast' sorCS1 was linear in time only for a relatively short period. Approximate shedding rates in the presence of PMA were therefore determined by adjusting incubation times, and separate experiments showed that the rates of sorCS1 shedding calculated from incubations for 7.5 and 15 min were indistinguishable, i.e. shedding was approximately linear for 15 min. Table 1 summarizes the results and shows that the stimulated rate of shedding of sorCS1 (and of the slightly 'slower' sorCS3) is two orders of magnitude higher than those of sorCS2 and sortilin, whereas sorLA holds an intermediate position.

Shed receptor binds ligands

We analysed ectodomains of the Vps10p-D receptors shed at appreciable rates. PDGF-BB is an established ligand of sorLA that binds to a soluble form of the receptor secreted from cells transfected with cDNA covering the entire luminal domain

Table 1 Rates of shedding of Vps10p-D receptors

CHO-K1 transfectants were incubated in fresh medium with or without PMA. Incubation times were chosen to ensure linear shedding (e.g. 15 min for stimulated sorCS1 transfectants; 2 h for unstimulated sortilin transfectants) followed by Western blotting of media and lysates, and quantification of receptor species by luminescence image analysis. Ectodomain released to the medium per min is expressed as percentage of full-length receptor present in cells incubated in the absence of PMA (i.e. steady-state content). The results are the mean values for four replicate incubations for each transfectant, and the coefficient of variation of the measurements was approx. 15%.

PMA	Ectodomain release (% per min $\times 10^2$)				
	sorCS1*	sorCS3	sorCS2	Sortilin	sorLA
–	61	29	1.0	0.3	6.8
+	209	103	1.2	0.9	48.2

*sorCS1c was taken as representative of the sorCS1 isoforms.

(soluble sorLA) [21]. Figure 9(A) shows that shed sorLA, purified by RAP affinity chromatography from culture medium of cells expressing the full-length receptor, binds this growth factor almost identically with soluble sorLA. The same picture emerged for several other established ligands of soluble sorLA: APP₆₉₅ ($K_d \sim 20$ nM) [15], apolipoprotein E3 ($K_d \sim 2$ nM) [20], complex of urokinase-type plasminogen activator and its type-1 inhibitor ($K_d \sim 4$ nM) [21] and RAP ($K_d \sim 2$ nM) [20]. Interestingly, the fragment APP-(304–612) bound to shed and soluble sorLA with an affinity ($K_d \sim 30$ nM) comparable with that of APP₆₉₅, indicating that the fragment harbours a major recognition motif for binding to the receptor. These results demonstrate that shed sorLA has a ligand binding profile very similar to that of the entire luminal domain.

Unlike sorLA, sorCS1 does not bind RAP or any other ligand tested until now [13], including APP (results not shown). We hypothesized that soluble sorCS1 might share the ability to bind PDGF-BB with sorLA, and Figure 9(B) shows that this is the case, whereas little binding was obtained with PDGF-CC. Other experiments (not demonstrated) showed that soluble sorCS3 binds PDGF-BB similarly to soluble sorCS1. Although shed sorCS1 and sorCS3 were not purified due to lack of a suitable affinity matrix, the results with sorLA released by TACE-mediated shedding suggest that the shed versions of sorCS receptors may also bind the growth factor.

DISCUSSION

The results demonstrate that ectodomains of all members of the Vps10p-D receptor family are shed constitutively and after PMA stimulation, although at very different rates: sorCS1 isoforms $>$ sorCS3 $>$ sorLA $\gg$ sorCS2 and sortilin. The TACE-mediated extracellular juxtamembrane cleavage elicits rapid degradation of the membrane-tethered stubs, whereas shed ectodomains are stable in the culture medium.

Members of the sorCS subfamily

Shedding of sorCS1 and sorCS3 in CHO-K1 cells is primarily mediated by TACE, since a marked reduction was observed in CHO-M2 cells deficient in active TACE, whereas sorCS2 is remarkably resistant to this proteinase. Previous studies have shown that extracellular cleavage of p75^{NTR} (designated α -secretase cleavage) is critically dependent on the position of the cleavage site with respect to the transmembrane domain. In fact, a stalk region of only 15 extracellular amino acids is required for efficient cleavage, and spacing of residues within this region is

Figure 9 Binding of PDGF-BB to shed and soluble sorLA, and to soluble sorCS1

Binding was measured by surface plasmon resonance analysis with 45 fmol/mm² immobilized receptor. (A) Binding of PDGF-BB to shed sorLA (solid lines) and soluble sorLA, i.e. the entire luminal domain (broken lines; only displayed at 20, 50 and 100 nM). The overall K_d values (global fitting) were calculated at 15 and 17 nM respectively. The inset shows the purity (silver gel) of shed (left) and soluble (right) sorLA. (B) Binding of PDGF-BB to soluble sorCS1 (solid lines). The K_d value was calculated at 30 nM. The broken line shows minor binding of 20 nM PDGF-CC. The inset shows purified soluble sorCS1.

more important than their identity [27]. As depicted in Figure 10, the sequences comprising the approx. 15-amino-acid juxtamembrane part of the stalk regions are similar in sorCS1 and sorCS3, but different in sorCS2. It may therefore be proposed that sorCS1 and sorCS3 are cleaved by TACE within their 15-amino-acid juxtamembrane regions, whereas the equivalent region in sorCS2 is resistant to the enzyme.

In contrast with CHO-K1 transfectants, SY5Y neuroblastoma cells exhibit minimal constitutive shedding of endogenous sorCS1c in spite of a high level of stimulated shedding. This suggests that release of luminal sorCS1 may be highly regulated in SY5Y cells. Marked differences in constitutive shedding between cell types have previously been noted for p75^{NTR} [9,10] and might be due to differences in activities of the implicated pathways. In addition, a different sheddase might be important in the SY5Y cells. In fact, whereas TACE/ADAM17 appears to be primarily responsible for α -secretase cleavage of APP in CHO cells, ADAM10 plays the central role in SY5Y cells [28,29],

Figure 10 Alignment of sorCS1, sorCS2 and sorCS3 extracellular stalk regions and transmembrane domains

The stalk regions and transmembrane domains (TM) are shown with amino acid numbers in parentheses, and residues in sorCS3 and sorCS2 identical with those in sorCS1 are indicated. The cds C-terminal to the KFKR signature are completely different for the sorCS1a, sorCS1b and sorCS1c isoforms. The cds of sorCS3 and sorCS2 both show 44% amino acid identity with the sorCS1c-cd. The luminal parts of mature sorCS3 and sorCS2 N-terminal to the displayed sequences (i.e. not including the propeptides) show 73 and 49% amino acid identity respectively to that of sorCS1.

and this may also apply to sorCS1, since the two proteinases can function redundantly.

After extracellular juxtamembrane cleavage, the membrane-tethered C-terminal fragment is rapidly degraded, since no accumulation of the sorCS1c stub occurs in membranes of cells incubated without additions, and cleavage within the membrane is important, as judged by accumulation of stub in the presence of the γ -secretase inhibitor DAPT. The extracellular cleavage is necessary for subsequent attack by γ -secretase-like activity because a high-molecular-mass form of sorCS1, expected to be mainly released to the medium if derived from constitutive intramembrane cleavage, was not observed when TACE activity was either inhibited by GM6001 or absent as in M2 cells. In contrast with sorCS2, sorCS1 isoforms and sorCS3 can therefore be regarded as subject to regulated (i.e. by removal of most of the luminal domain) intramembrane proteolysis. Although future studies should determine the nature of the γ -secretase, members of the presenilin-type aspartyl proteinase family are likely candidates, since they cleave type-I membrane proteins conditioned by prior truncation [5]. In any case, the transmembrane domain of sorCS1, which is almost identical with that of sorCS3 (Figure 10), is a good substrate for the γ -secretase involved. Interestingly, the transmembrane domain of sorCS2 is completely different and, paradoxically, reminiscent of that of p75^{NTR}, which is cleaved by a presenilin-dependent γ -secretase within the sequence AAVV↓VGLV in the middle of the domain [11]. It is therefore possible that sorCS2 remains uncleaved by γ -secretase activity because the ectodomain shedding is inefficient.

Sortilin and sorLA

Previous studies have reported constitutive and phorbol ester-stimulated shedding of sortilin [30] and sorLA [21,31,32] in various cell types, including CHO-K1 transfectants, but the convertase involved was not determined. The present study shows that TACE-mediated shedding of sortilin does occur in CHO-K1 cells, but at very low rates as compared with sorCS1 isoforms, indicating that the extracellular juxtamembrane part of sortilin is a poor substrate for TACE. On the other hand, TACE-mediated shedding of sorLA occurs at appreciable rates. The resulting stub seems more resistant to degradation than that of sorCS1c, since small amounts can be detected even in the absence of DAPT, and accumulation is marked in the presence of the inhibitor. As for sorCS1, we were unable to detect the cd generated by cleavage of the stub within the membrane, and we assume that it is rapidly degraded.

Possible functional implications

In general, ectodomain shedding causes down-modulation of the full-length receptor and generation of a soluble fragment plus a membrane-tethered stub. Even when shedding of Vps10p-D receptors occurs at high rates, cleavage of the stubs follows the same pace, and cds should therefore be generated at high rates.

Although the possibility cannot be excluded, signalling functions of the released cds seem unlikely, because we were unable to detect any accumulation.

The generation of shed sortilin appears limited as judged from the experiments with CHO-K1 transfectants. This receptor binds p75^{NTR}, and a death signalling complex is formed by cross-binding of proNGF whose propeptide binds to sortilin [16]. A similar signalling complex is formed by cross-binding of proBDNF (pro-brain-derived neurotrophic factor), and full-length sortilin is required to initiate cell death, whereas soluble sortilin is antagonistic [33]. p75^{NTR}, as well as its stub generated by TACE, also interacts with the TrkA receptor, whereas the p75^{NTR}-cd does not, suggesting that p75^{NTR} cleavage is involved in regulation of TrkA receptor activity [11]. By analogy, this may also apply to the TrkB receptor. It will therefore be interesting to clarify if binding to sortilin can influence the stability of p75^{NTR} and thereby its ability to form heteromeric receptor complexes.

Blockade of sorLA shedding is reported to decrease proliferation of cultured neuronal cells [31], and conditioned medium containing shed sorLA increases migration of smooth muscle cells in the presence of PDGF-BB [32]. We show that purified shed sorLA binds PDGF-BB, as previously reported for soluble sorLA comprising the entire luminal domain of the receptor [21]. The results suggest that the complex of PDGF-BB and shed sorLA may promote signalling via the PDGF- β receptor, in analogy with the established ability of interleukin-6 and its soluble receptor- α to promote signalling via the membrane-associated receptor gp130 [34]. Several other ligands also showed similar binding to the two sorLA versions, demonstrating that shed sorLA can function as a binding protein in a wider context.

Until now, no ligand has been identified for sorCS1, and the meaning of its high rate of shedding therefore remains elusive. SorCS1 and SorCS3 are both expressed in the hippocampus and are up-regulated by kainic acid-induced seizures [14]. We show that the luminal domains of these receptors bind PDGF-BB similarly to soluble and shed sorLA and, by analogy, this is likely to apply also to the shed sorCS versions. PDGF-BB attenuates excitotoxic death of cultured hippocampal neurons [35] and promotes proliferation and migration of newly born cells after intraventricular administration [36], effects that are likely to be modified by binding proteins. Interestingly, soluble sorCS3 [17] and sorCS1 (G. Hermey and J. Gliemann, unpublished work), also bind NGF propeptide, although with an affinity somewhat lower than that of sortilin, suggesting that shed versions of sorCS receptors may have wider functions as growth factor-binding proteins.

In summary, we show that sorCS1 and sorCS3 are shed at high rates, sorCS2 and sortilin at low rates, and sorLA at intermediate rates. Except for sorCS2, TACE is the major enzyme responsible for the shedding events, which are followed by rapid cleavage of the membrane-tethered stubs. We show that shed sorLA is capable of binding ligands and propose that shed versions of a subset of Vps10p-D receptors serve as binding proteins for several ligands, including growth factors.

This work was supported by grants from the Danish Medical Research Council, the Novo Nordic Foundation, the Alfred Benzon Foundation and the Lundbeck Foundation. We thank Dr Joaquín Arribas for CHO-M2 cells and Dr Roberto Cappai for APP₆₉₅. Dr C. Jacobsen (Institute of Medical Biochemistry, University of Aarhus) is thanked for help with surface plasmon resonance analyses and Anja Aagaard for excellent technical assistance.

REFERENCES

- Hooper, N. M., Karran, E. H. and Turner, A. J. (1997) Membrane protein secretases. *Biochem. J.* **321**, 265–279
- Dello Sbarba, P. and Rovida, E. (2002) Transmodulation of cell surface regulatory molecules via ectodomain shedding. *Biol. Chem.* **383**, 69–83
- Arribas, J. and Merlos-Suarez, A. (2003) Shedding of plasma membrane proteins. *Curr. Top. Dev. Biol.* **54**, 125–144
- Blobel, C. P. (2005) ADAMS: key components in EGFR signalling and development. *Nat. Rev. Mol. Cell Biol.* **6**, 32–43
- Landman, N. and Kim, T.-W. (2004) Got RIP? Presenilin-dependent intramembrane proteolysis in growth factor receptor signaling. *Cytokine Growth Factor Rev.* **15**, 337–351
- Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J. R., Cumano, A., Roux, P., Black, R. A. and Israel, A. (2000) A novel proteolytic cleavage involved in notch signaling: the role of the disintegrin-metalloprotease TACE. *Mol. Cell* **5**, 207–216
- Mumm, J. S., Schroeter, E. H., Saxena, M. T., Griesemer, A., Tian, X., Pan, D. J., Ray, W. J. and Kopan, R. (2000) A ligand-induced extracellular cleavage regulates γ -secretase-like proteolytic activation of notch1. *Mol. Cell* **5**, 197–206
- Schroeter, E. H., Kisslinger, J. A. and Kopan, R. (1998) Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature (London)* **393**, 382–386
- Kanning, K. C., Hudson, M., Amieux, P. S., Wiley, J. C., Bothwell, M. and Schecterson, L. C. (2003) Proteolytic processing of the p75 neurotrophin receptor and two homologs generates C-terminal fragments with signaling capability. *J. Neurosci.* **23**, 5425–5436
- Weskamp, G., Schlöndorff, J., Lum, L., Becherer, J. D., Kim, T.-W., Saftig, P., Hartmann, D., Murphy, G. and Blobel, C. P. (2004) Evidence for a critical role of the tumor necrosis factor α convertase (TACE) in ectodomain shedding of the p75 neurotrophin receptor (p75^{NTR}). *J. Biol. Chem.* **279**, 4241–4249
- Jung, K.-M., Tan, S., Landman, N., Petrova, K., Murray, S., Lewis, R., Kim, P. K., Kim, D. S., Ryu, S. H., Chao, M. V. et al. (2003) Regulated intramembrane proteolysis of the p75 neurotrophin receptor modulates its association with the TrkA receptor. *J. Biol. Chem.* **278**, 42161–42169
- Westergaard, U. B., Sørensen, E. S., Hermey, G., Nielsen, M. S., Nykjær, A., Kirkegaard, K., Jacobsen, C., Gliemann, J., Madsen, P. and Petersen, C. M. (2004) Functional organization of the sortilin Vps10p domain. *J. Biol. Chem.* **279**, 50221–50229
- Hermey, G., Keat, S. J., Madsen, P., Jacobsen, C., Petersen, C. M. and Gliemann, J. (2003) Characterization of sorCS1, an alternatively spliced receptor with completely different cytoplasmic domains that mediate different trafficking in cells. *J. Biol. Chem.* **278**, 7390–7396
- Hermey, G., Plath, N., Hubner, C. A., Kuhl, D., Schaller, H. C. and Hermans-Borgmeyer, I. (2004) The three sorCS genes are differentially expressed and regulated by synaptic activity. *J. Neurochem.* **88**, 1470–1476
- Andersen, O. M., Reiche, J., Schmidt, V., Gotthardt, M., Spoelgen, R., Behlke, J., von Arnim, C. A. F., Breiderhoff, T., Jansen, P., Wu, X. et al. (2005) SorLA/LR11, a neuronal sorting receptor that regulates processing of the amyloid precursor protein. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 13461–13466
- Nykjaer, A., Lee, R., Teng, K. K., Jansen, P., Madsen, P., Nielsen, M. S., Jacobsen, C., Klemmannel, M., Schwarz, E., Willnow, T. E. et al. (2004) Sortilin is essential for proNGF-induced neuronal cell death. *Nature (London)* **427**, 843–848
- Westergaard, U. B., Kirkegaard, K., Sørensen, E. S., Jacobsen, C., Nielsen, M. S., Petersen, C. M. and Madsen, P. (2005) SorCS3 does not require propeptide cleavage to bind nerve growth factor. *FEBS Lett.* **579**, 1172–1176
- Mazella, J., Zsuzser, N., Navarro, V., Chabry, C. J., Kaghad, M., Caput, D., Ferrara, P., Vita, N., Gully, D., Maffrand, J. P. et al. (1998) The 100-kDa neurotensin receptor is gp95/sortilin, a non-G-protein-coupled receptor. *J. Biol. Chem.* **273**, 26273–26276
- Petersen, C. M., Nielsen, M. S., Jacobsen, C., Tauris, J., Jacobsen, L., Gliemann, J., Moestrup, S. K. and Madsen, P. (1999) Propeptide cleavage conditions sortilin/neurotensin receptor-3 for ligand binding. *EMBO J.* **18**, 595–604
- Jacobsen, L., Madsen, P., Jacobsen, C., Nielsen, M. S., Gliemann, J. and Petersen, C. M. (2001) Activation and functional characterization of the mosaic receptor sorLA/LR11. *J. Biol. Chem.* **276**, 22788–22796
- Gliemann, J., Hermey, G., Nykjær, A., Petersen, C. M., Jacobsen, C. and Andreasen, P. A. (2004) The mosaic receptor sorLA/LR11 binds components of the plasminogen-activating system and platelet-derived growth factor-BB similarly to LRP1 (low-density lipoprotein receptor-related protein), but mediated slow internalization of bound ligand. *Biochem. J.* **381**, 203–212
- Nielsen, M. S., Madsen, P., Christensen, E. I., Nykjær, A., Gliemann, J., Kasper, D., Pohlmann, R. and Petersen, C. M. (2001) The sortilin cytoplasmic tail conveys Golgi-endosome transport and binds the VHS domain of the GGA2 sorting protein. *EMBO J.* **20**, 2180–2190
- Borroto, A., Ruiz-Paz, S., de la Torre, T. V., Borrell-Pages, M., Merlos-Suarez, A., Pandiella, A., Blobel, C. P., Basalga, J. and Arribas, J. (2003) Impaired trafficking and activation of tumor necrosis factor- α -converting enzyme in cell mutants defective in ectodomain shedding. *J. Biol. Chem.* **278**, 25933–25939
- Villanueva de la Torre, T., Bech-Serra, J. J., Ruiz-Paz, S., Basalga, J. and Arribas, J. (2004) Inactivating mutants block the tumor necrosis factor- α -converting enzyme in the early secretory pathway. *Biochem. Biophys. Res. Commun.* **314**, 1028–1035
- Li, X. and Fan, H. (2004) Loss of ectodomain shedding due to mutations in the metalloprotease and cysteine-rich/disintegrin domains of the tumor necrosis factor- α converting enzyme. *J. Biol. Chem.* **279**, 27365–27375
- Guan, R., Zhang, Y., Jiang, J., Baumann, C. A., Black, R. A., Baumann, G. and Frank, S. J. (2001) Phorbol ester- and growth factor-induced growth hormone (GH) receptor proteolysis and GH-binding protein shedding: relationship to GH receptor down-regulation. *Endocrinology* **142**, 1137–1147
- Zampieri, N., Xu, C., Neubert, T. A. and Chao, M. V. (2005) Cleavage of p75 neurotrophin receptor by α -secretase and γ -secretase requires specific receptor domains. *J. Biol. Chem.* **280**, 14563–14571
- Allinson, T. M. J., Parkin, E. T., Turner, A. J. and Hooper, N. M. (2003) ADAMs family members as amyloid precursor protein α -secretases. *J. Neurosci. Res.* **74**, 342–352
- Allinson, T. M. J., Parkin, E. T., Condon, T. P., Schwager, S. L., Sturrock, E. D., Turner, A. J. and Hooper, N. M. (2004) The role of ADAM10 and ADAM17 in the ectodomain shedding of angiotensin converting enzyme and the amyloid precursor protein. *Eur. J. Biochem.* **271**, 2539–2547
- Navarro, V., Vincent, J.-P. and Mazella, J. (2002) Shedding of the luminal domain of the neurotensin receptor-3/sortilin in the HT29 cell line. *Biochem. Biophys. Res. Commun.* **298**, 760–764
- Hampe, W., Riedel, I. B., Lintzel, J., Bader, C. O., Franke, I. and Schaller, H. C. (2000) Ectodomain shedding, translocation and synthesis of sorLA are stimulated by its ligand head activator. *J. Cell Sci.* **113**, 4475–4485
- Zhu, Y., Bujo, H., Yamazaki, H., Ohwaki, K., Jiang, M., Hirayama, S., Kanaki, T., Shibasaki, M., Takahashi, K., Schneider, W. J. et al. (2004) LR11, an LDL receptor gene family member, is a novel regulator of smooth muscle cell migration. *Circ. Res.* **94**, 752–758
- Teng, H. K., Teng, K. K., Lee, R., Wright, S., Tevar, S., Almeida, R., Kermani, P., Torkin, R., Chen, Z.-Y., Lee, F. S. et al. (2005) ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. *J. Neurosci.* **25**, 5455–5463
- Gomez, M. L., Sokol, S. H., Muir, A. B., Soong, G., Bastien, J. and Prince, A. S. (2005) Bacterial induction of TNF- α converting enzyme expression and IL-6 receptor- α shedding regulates airway inflammatory signaling. *J. Immunol.* **175**, 1930–1936
- Tseng, H. C. and Dichter, M. A. (2005) Platelet-derived growth factor-BB pretreatment attenuates excitotoxic death in cultured hippocampal neurons. *Neurobiol. Dis.* **19**, 77–83
- Mohapel, P., Frielingsdorf, H., Häggblad, J., Zachrisson, O. and Brundin, P. (2005) Platelet-derived growth factor (PDGF-BB) and brain-derived neurotrophic factor (BDNF) induce striatal neurogenesis in adult rats with 6-hydroxydopamine lesions. *Neuroscience* **132**, 767–776

Received 19 August 2005/5 December 2005; accepted 4 January 2006

Published as BJ Immediate Publication 4 January 2006, doi:10.1042/BJ20051364