

# Propeptide cleavage conditions sortilin/neurotensin receptor-3 for ligand binding

C.Munck Petersen<sup>1</sup>, M.S.Nielsen,  
C.Jacobsen, J.Tauris, L.Jacobsen,  
J.Gliemann, S.K.Moestrup and P.Madsen

Department of Medical Biochemistry, University of Aarhus,  
Ole Worms Allé, Building 170, DK-8000 Aarhus C, Denmark

<sup>1</sup>Corresponding author  
e-mail: CMP@biokemi.au.dk

**We recently reported the isolation and sequencing of sortilin, a new putative sorting receptor that binds receptor-associated protein (RAP). The luminal N-terminus of sortilin comprises a consensus sequence for cleavage by furin, R<sup>41</sup>WRR<sup>44</sup>, which precedes a truncation originally found in sortilin isolated from human brain. We now show that the truncation results from cellular processing. Sortilin is synthesized as a proform which, in late Golgi compartments, is converted to the mature receptor by furin-mediated cleavage of a 44 residue N-terminal propeptide. We further demonstrate that the propeptide exhibits pH-dependent high affinity binding to fully processed sortilin, that the binding is competed for by RAP and the newly discovered sortilin ligand neurotensin, and that prevention of propeptide cleavage essentially prevents binding of RAP and neurotensin. The findings evidence that the propeptide sterically hinders ligands from gaining access to overlapping binding sites in prosortilin, and that cleavage and release of the propeptide preconditions sortilin for full functional activity. Although proteolytic processing is involved in the maturation of several receptors, the described exposure of previously concealed ligand-binding sites after furin-mediated cleavage of propeptide represents a novel mechanism in receptor activation.**

*Keywords:* furin/neurotensin/propeptide/RAP/sortilin

## Introduction

We have previously reported the purification and cDNA cloning of sortilin, a new putative 95 kDa sorting receptor of human brain (Petersen *et al.*, 1997). Sortilin is a typical type-1 membrane protein, consisting of a large luminal domain, a single transmembrane segment and a short C-terminal cytoplasmic tail. Both the luminal and the cytoplasmic domains bear a distinct and indicative resemblance to well known sorting receptors (Petersen *et al.*, 1997). The luminal domain is similar to each of the two domains that constitute the luminal part of Vps10p, a yeast receptor for carboxypeptidase Y sorting (Marcusson *et al.*, 1994), and the C-terminal segment of sortilin's cytoplasmic tail is identical to the corresponding segment in the cation-independent mannose 6-phosphate/insulin

growth factor-II receptor (CI-M6PR). Overall, the sortilin tail contains several well known sorting motifs, suggesting that the receptor has the potential to engage in various types of trafficking, including endocytosis and transport between the Golgi and late endosomes. Only a minor fraction of the sortilin receptors are normally present at the cell surface (Mazella *et al.*, 1998; Morris *et al.*, 1998) and although recent findings have shown that certain stimuli can up-regulate expression on the surface membrane, e.g. insulin in 3T3-L1 adipocytes (Morris *et al.*, 1998) and neurotensin in embryonic neurons (Chabry *et al.*, 1993), the major pool of sortilin and sortilin tail hybrid receptors accumulate in the Golgi and in vesicles, co-localizing with the CI-M6PR (Petersen *et al.*, 1997; Morris *et al.*, 1998). Thus, sortilin's subcellular localization seems to agree with its suggested role as a sorting receptor.

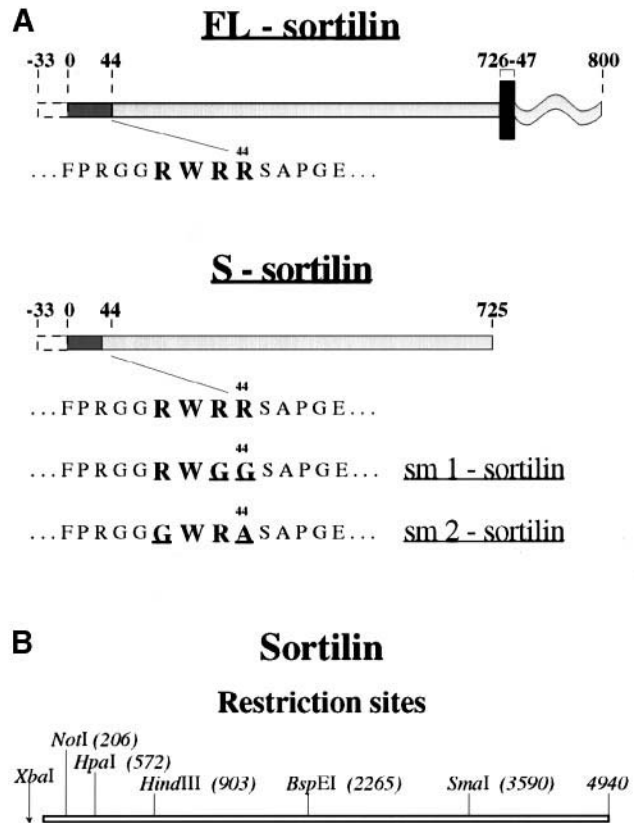
So far two sortilin ligands have been identified, firstly the receptor-associated protein (RAP) used for the original affinity purification of sortilin from human brain, and secondly the neuropeptide neurotensin. RAP is a 40 kDa endoplasmic reticulum (ER)-resident protein which binds to members of the low density lipoprotein receptor (LDLR) family of multifunctional endocytic receptors. This family of receptors, in particular the giant receptors LRP (low density lipoprotein receptor-related protein) and gp330/megalin, covers a diversity of ligands which bind to separate sites, but it is characteristic that binding of all ligands is inhibited by RAP (for review see Gliemann, 1998). Recent studies strongly indicate that RAP serves as a chaperone for members of the LDLR family by protecting them from aggregation caused by premature binding of multisite ligands in the synthetic pathway, and possibly by assisting in the folding of the receptors (for reviews see Bu and Schwartz, 1998; Willnow, 1998). An additional brain receptor, sorLA-1, which we isolated in parallel with sortilin (Jacobsen *et al.*, 1996), has similarities to sortilin and also binds RAP. However, sorLA-1 contains a number of the typical structural elements of the LDLR, including the ligand-binding class A cluster repeats, and sortilin is therefore the only known RAP-binding receptor without similarities to the LDLR family.

In contrast to RAP, neurotensin is secreted into the extracellular environment and has several well described functions. Along with its analogue neuromedin-N, neurotensin is synthesized as part of 147 amino acid precursor protein (Dobner *et al.*, 1987; Bean *et al.*, 1992) and is released in secretory vesicles as a bioactive 13 amino acid peptide upon cleavage of the precursor by members of the subtilisin/Kex2-like family of mammalian prohormone convertases (Barbero *et al.*, 1998). The peptide is produced by neurons and induces several effects in the central nervous system (CNS), such as an increase in dopamine turnover, hypothermia and muscle relaxation. It is also synthesized outside the CNS, and peripheral administration

has been shown to cause hyperglycaemia, hypo- or hypertension as well as histamine release (for review see Vincent, 1995). Neurotensin's effects are mediated by signalling membrane receptors, and two such receptors, both typical G-protein-associated receptors, have been cloned in recent years (Tanaka *et al.*, 1990; Chalon *et al.*, 1996). Sortilin has now been identified as the third neurotensin-binding receptor, and it seems to be the first non-G-protein-coupled receptor that binds a neuropeptide (Mazella *et al.*, 1998). There is nothing to indicate that sortilin is a signalling receptor, but previous findings strongly suggest that sequestration of G-protein-coupled receptors in response to neurotensin stimulation is accompanied by translocation of intracellular sortilin to the cell surface where it may bind and internalize neurotensin (Chabry *et al.*, 1993). Thus, it is likely that sortilin participates in regulating the turnover of neurotensin.

Sortilin purified from brain by either RAP or neurotensin affinity chromatography carries an N-terminal truncation covering 44 residues. Moreover, the sequence R<sup>41</sup>WRR<sup>44</sup> immediately preceding the N-terminus of the truncated receptor is identical to the consensus sequence for cleavage by the cellular endoproteinase furin (Lin *et al.*, 1997; Petersen *et al.*, 1997; Mazella *et al.*, 1998; Morris *et al.*, 1998). Furin is one of the seven currently known homologous proteinases that constitute the family of proprotein-converting enzymes (PCs) in the synthetic and vesicular pathway of mammalian cells (for reviews see Nakayama, 1997; Seidah and Chrétien, 1997). Conversion by cleavage of newly synthesized proteins is an essential part of processing, and accounts for the activation/generation of a multitude of cellular proteins including peptide hormones, enzymes, coagulation factors and cell signalling molecules. Some of the PCs have a limited tissue expression, but furin and PC7, both transmembrane enzymes that are located in the *trans*-Golgi network (TGN) and constitutive secretory vesicles, are widespread and appear to be expressed in all normal cell types. The PCs all cleave at single or pairs of basic residues, and their functions may overlap to some extent. However, their distribution and cleavage sequence preference differ. Therefore, they cannot substitute for each other and, accordingly, furin knockouts appear to result in embryonic death (Seidah and Chrétien, 1997). Whereas conversion of secretory proteins is a very common event, cleavage as part of receptor processing seems to be an exception rather than the rule. The few receptors that presently are known to be subject to PC-mediated cleavage, e.g. the insulin receptor, the insulin-like growth factor-1 receptor, the hepatocyte growth factor receptor and the LRP, are all produced as single chain proreceptors (precursors) that are converted by furin into one  $\alpha$ - and one  $\beta$ -subunit, which constitute the mature receptors (Komada *et al.*, 1993; Bravo *et al.*, 1994; Willnow *et al.*, 1996a; Lehmann *et al.*, 1998). Precursor cleavage does not directly involve the ligand-binding sites of the receptors, and the precursors of the hepatocyte growth factor receptor and LRP both seem to exhibit almost normal functional activity.

The present study was undertaken to establish the mechanism and location of prosortilin cleavage and to determine if the truncation influences the ligand binding of sortilin.



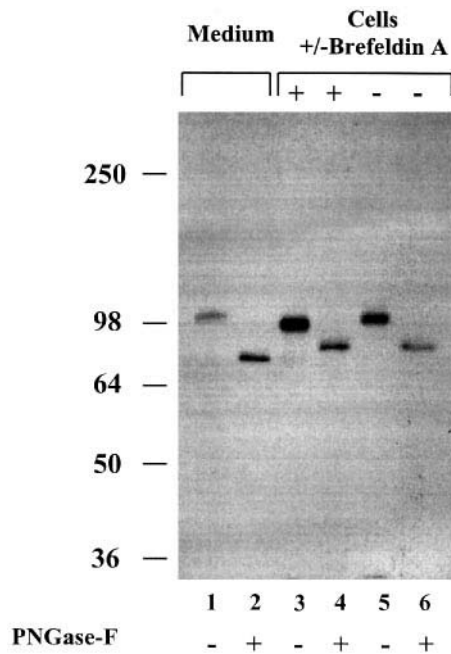
**Fig. 1.** (A) Schematic presentation of the sortilin constructs used in this study, the full-length receptor (fl-sortilin) and the three soluble minireceptors (s-sortilin, sm1- and sm2-sortilin). The leader peptide (-33 to -1), the propeptide (1-44; dark grey), the luminal domain (45-725; light grey), the transmembrane segment (726-747; black) and the cytoplasmic tail (748-800) are indicated with reference to flanking amino acid residues. The sequence containing the propeptide consensus site (upper case letters) for cleavage by furin is given. Altered residues in the soluble mutant minireceptors are underlined. (B) Map of the restriction sites in the sequenced sortilin strand used for generating the various cDNA constructs.

## Results

Initially, a soluble sortilin minireceptor (s-sortilin, Figure 1), comprising the luminal, RAP-binding domain of sortilin, but not its cytoplasmic tail or transmembrane segment, was expressed in CHO-K1 cells. The receptor was purified from the culture medium by RAP affinity chromatography and shown by microsequencing to have the same N-terminus as the truncated sortilin originally isolated from human brain (Petersen *et al.*, 1997; Tauris *et al.*, 1998). This suggested that wild-type sortilin is synthesized as a proform and converted to the mature receptor by cleavage of a 44 amino acid propeptide. The following experiments were designed to confirm this mechanism and to elucidate the location and the mechanism of the cleavage.

### ***The sortilin precursor is cleaved during or after passage through the TGN***

To establish that a truncation had taken place during processing, the CHO transfectants were biolabelled, and s-sortilin secreted into the medium or contained in the cell lysates was immunoprecipitated. In parallel, s-sortilin was precipitated from labelled transfectants cultured in

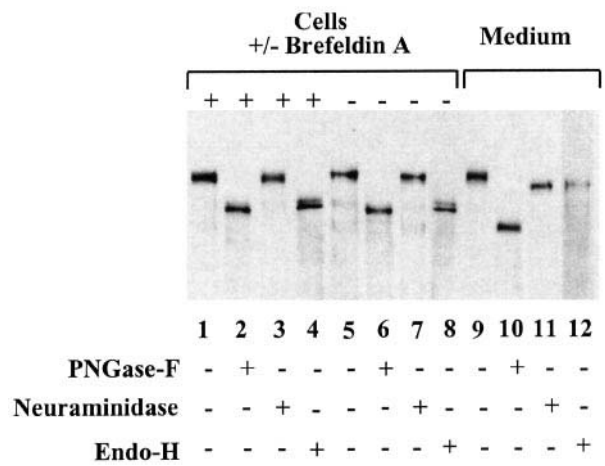


**Fig. 2.** Soluble sortilin minireceptor (s-sortilin) in transfected cells. CHO-K1 cells, stably transfected with soluble sortilin (s-sortilin), were biolabelled with Pro-mix in cysteine and methionine-free medium. After 4 h, the medium was recovered and the cells were washed and lysed in lysis buffer containing proteinase inhibitors. Sortilin was immunoprecipitated from the medium (lanes 1 and 2) and from lysates of cells cultured in the presence (lanes 3 and 4) or absence (lanes 5 and 6) of brefeldin A. The precipitates were analysed by SDS-PAGE before and after treatment with PNGase-F. An autoradiograph of an 8% polyacrylamide PPO-impregnated gel is shown. Medium from brefeldin A-treated cells contained no s-sortilin and is not included.

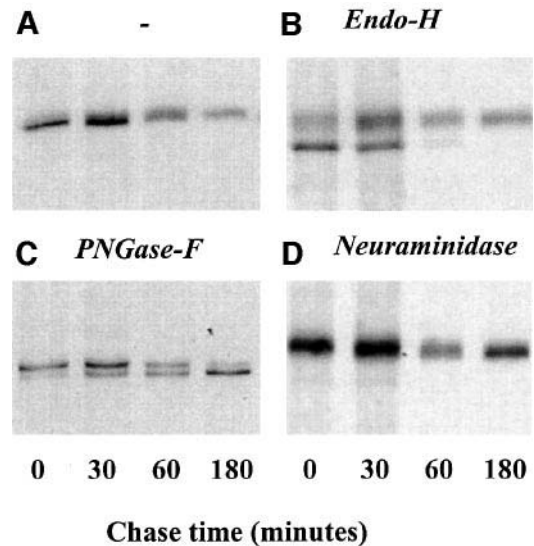
the presence of brefeldin A, a drug that retains newly synthesized proteins in the ER and prevents their secretion (Misumi *et al.*, 1986). Figure 2 shows an SDS-PAGE analysis of the precipitates and, as can be seen from the migration pattern, the truncated receptor obtained from the medium (lane 1) presents a similar or higher molecular weight than each of the two cellular species (lanes 3 and 5). Further analysis showed that this apparent paradox is accounted for by differential glycosylation. Thus, after removal of sugars by *N*-glycosidase-F (PNGase-F), s-sortilin from the medium (Figure 2, lane 2) migrates in accordance with a molecular weight ~5 kDa lower than that of deglycosylated receptors originating from cell lysates (lanes 4 and 6). The results demonstrate that secreted s-sortilin is truncated, whereas cellular s-sortilin represents uncleaved proforms. It follows that conversion of pro-s-sortilin is likely to take place immediately before its exit from the cell.

To determine further the site of conversion, the glycosylation of s-sortilin was also analysed using Endo-H and neuraminidase. The results in Figure 3 demonstrate that each of the two cellular species, from brefeldin A-treated and untreated cells, were sensitive to Endo-H, but insensitive to treatment with neuraminidase. In contrast, the secreted receptor isolated from medium was much less sensitive to Endo-H (Figure 3, lane 12 versus lanes 4 and 8), but clearly presents a shift in electrophoretic mobility upon incubation with neuraminidase (lane 11 versus lanes 3 and 7), signifying a loss of sialic acids.

Results were similar in CHO cells transfected with full-



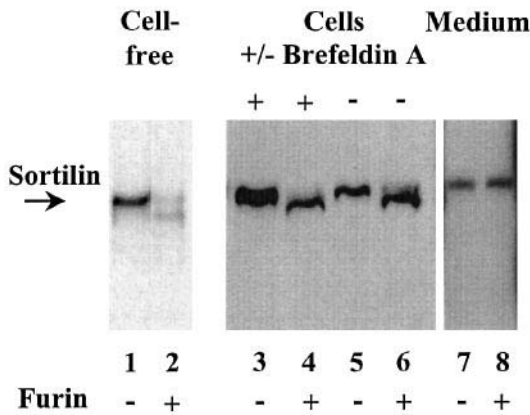
**Fig. 3.** Deglycosylation of s-sortilin. The figure shows an analysis of biolabelled s-sortilin produced in transfected CHO-K1 cells. Sortilin was immunoprecipitated from lysates of brefeldin A-treated cells (lanes 1–4), and from lysates (lanes 5–8) and medium (lanes 9–12) of untreated cells. The precipitated protein subsequently was incubated (18 h, 30°C) without glycosidase (lanes 1, 5 and 9) or in the presence of either PNGase-F (lanes 2, 6 and 10), neuraminidase (lanes 3, 7 and 11) or Endo-H (lanes 4, 8 and 12).



**Fig. 4.** Deglycosylation and cleavage of stably expressed full-length sortilin. Transfected CHO-K1 cells expressing full-length sortilin were biolabelled for 1 h prior to chase in full medium, starting at zero time. At the indicated times, cells were lysed in the presence of EDTA and proteinase inhibitors. Sortilin, either untreated or after overnight incubation with Endo-H, PNGase-F or neuraminidase, was then immunoprecipitated and analysed by 8% SDS-PAGE.

length sortilin (fl-sortilin). Figure 4 shows an experiment in which the cells were biolabelled for 1 h and then subjected to a 3 h chase. At the times indicated, the cells were lysed and, after treatment with the given glycosidase, immunoprecipitated fl-sortilin was analysed by reducing SDS-PAGE. It appears from Figure 4C and D that propeptide cleavage of fl-sortilin coincides with the acquisition of sialic acids, i.e. an increased sensitivity to neuraminidase. Moreover, as only half of the receptor population has been cleaved at a point when Endo-H resistance is complete (Figure 4B and C, 60 min), cleavage occurs after the acquisition of Endo-H resistance.

The results above establish that sortilin is synthesized



**Fig. 5.** Cleavage of s-sortilin by furin. Sortilin, expressed in cell-free medium (lanes 1 and 2) or present in the medium (lanes 7 and 8) and lysates (lanes 3–6) of biolabelled cultures of stably transfected CHO-K1 cells, was reacted with furin (2 h, 37°C) in a  $\text{Ca}^{2+}$ -containing phosphate buffer or incubated under similar conditions in the absence of furin. For sortilin samples originating from cell-free expression, incubation with furin was stopped by addition of reducing SDS sample buffer. Reactions involving sortilin produced in cells were stopped by addition of 10 mM EDTA, followed by immunoprecipitation of sortilin and resuspension in sample buffer. The samples were loaded onto an 8% polyacrylamide gel and bands were visualized by autoradiography of PPO-impregnated gels.

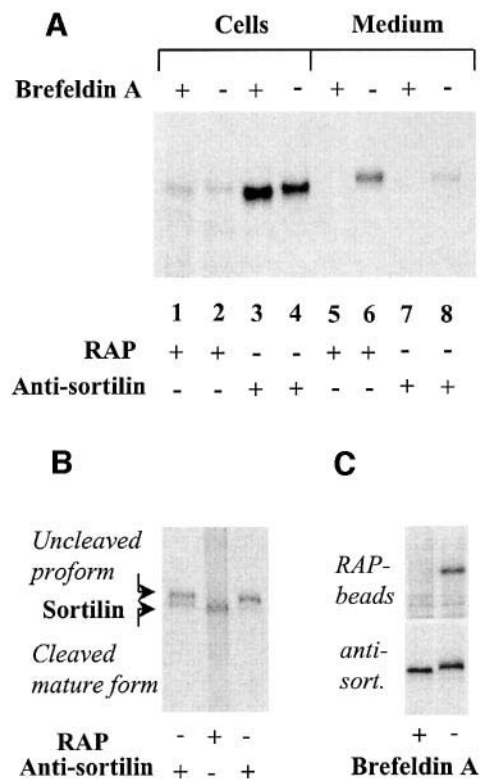
and leaves the ER as a proform, which is converted to the mature receptor by processing in late Golgi compartments (the TGN) and perhaps in post-Golgi vesicles.

The sequence immediately preceding the sortilin cleavage site ( $\text{R}^{41}\text{XRR}^{44}$ ; Figure 1) constitutes a consensus sequence for cleavage by the ubiquitous proprotein convertase furin, which resides in both the TGN and constitutive secretory vesicles, the site for conversion of pro-sortilin. Since initial experiments demonstrated that pro-s-sortilin translated in cell-free medium was at least partially cleaved upon co-incubation with recombinant furin (Figure 5, lanes 1 and 2), we next examined the outcome of co-incubations between furin and the three species of s-sortilin isolated from lysates and medium of CHO transfectants. As expected, furin did not alter the electrophoretic mobility of secreted s-sortilin (Figure 5, lanes 7 and 8), signifying that the fully processed receptor is not subject to additional cleavage. In contrast, s-sortilin from lysates of brefeldin A-treated as well as untreated cells was completely converted to faster migrating species by furin (Figure 5, lanes 3–6).

#### Propeptide cleavage elicits functional ligand binding

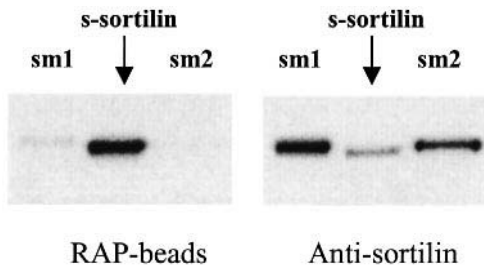
Despite the fact that cells hold pro- as well as mature sortilin, purified sortilin prepared from tissues by RAP affinity chromatography did not contain detectable proreceptor. The following experiments were therefore carried out to determine if ligand binding depends on proreceptor cleavage.

Figure 6A shows an experiment with biolabelled s-sortilin from cultures of CHO transfectants. After labelling, the content of RAP-binding s-sortilin in the medium and lysates was assayed by precipitation with RAP affinity beads. Unbound sortilin was quantified subsequently by immunoprecipitation using anti-sortilin antibody and GammaBind G beads. The precipitates were analysed



**Fig. 6.** (A) Precipitation of secreted and intracellular s-sortilin by RAP beads and anti-sortilin antibody. CHO-K1 cells expressing s-sortilin were biolabelled in the absence or presence of brefeldin. Following 4 h of incubation, the culture medium was harvested and the cells were lysed in lysis buffer containing EDTA and proteinase inhibitors. The recovered medium and the cell lysate were supplemented with lysis buffer and fresh medium, respectively, giving a similar composition of medium and lysis buffer (0.1% Triton X-100) in both samples. After pre-clearance with blank Sepharose beads, RAP-bindable protein was precipitated by overnight incubation with RAP-Sepharose beads (4°C), and unbound RAP subsequently was precipitated by immunoprecipitation. (B) RAP selectively binds cleaved sortilin. Biolabelled CHO-K1 cells were lysed and the medium was recovered. The cell lysate was then mixed with the original medium and sortilin was subjected to precipitation by anti-sortilin beads (lane 1) or by first RAP beads (lane 2) and then anti-sortilin beads (lane 3). The precipitated protein was analysed by SDS–8% PAGE and the PPO-impregnated gel was exposed to film at –80°C. (C) Binding of full-length sortilin. Full-length sortilin was stably expressed in CHO-K1 cells. The cells were biolabelled using ProMix and a medium without methionine. After 4 h of labelling, the medium was recovered, and the cells were washed in a balanced salt solution and lysed in lysis buffer containing EDTA and proteinase inhibitors. Sortilin was precipitated from the lysate by incubation with first RAP beads (upper panel, lane 2) and then anti-sortilin beads (lower panel, lane 2), and analysed by SDS–PAGE. Similar results obtained in cells labelled in the presence of brefeldin A are shown in the left lanes (1). The gel (8%) was treated with PPO and exposed to film at 80°C.

by reducing PAGE (Figure 6A) and, as determined by densitometry, ~84% of the secreted s-sortilin bound RAP (lanes 6 and 8). In contrast, <8% of the receptors contained in the cell lysates were RAP bindable, i.e. ~7.7% in brefeldin A-treated (Figure 6A, lanes 1 and 3) and ~7.6% in untreated cells (lanes 2 and 4). To ensure equal binding conditions for secreted and cellular s-sortilin, we performed an additional experiment in which medium containing cleaved receptor and cell lysate containing proreceptor were mixed prior to precipitation by RAP or antibodies. The precipitated protein was treated with



**Fig. 7.** Precipitation of soluble mutant receptors and s-sortilin by RAP beads and antibodies. CHO-K1 transfectants expressing the mutant sm2-sortilin (lanes 1 and 3) or s-sortilin (lanes 2 and 4) were biolabelled and the secreted minireceptors were precipitated from the medium by incubation with RAP beads. Receptors not bound by RAP subsequently were precipitated using anti-sortilin antibodies. The precipitates were analysed by reducing SDS-8% PAGE. The figure shows an autoradiography of a diphenyloxazole-fluorographed gel.

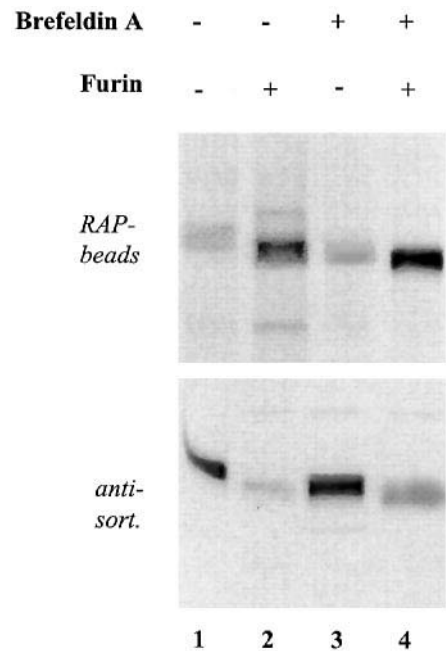
PNGase-F to allow electrophoretic distinction between cleaved and uncleaved s-sortilin. Figure 6B shows that although the mixture contained both types of s-sortilin (lane 1), only the cleaved, 'fast' form receptor was precipitated with RAP beads (lanes 2) whereas the 'slow' form remained in the medium and was precipitated by anti-sortilin antibody (lane 3).

Results were qualitatively similar using cells expressing fl-sortilin (Figure 6C). Under normal culture conditions, a significant fraction of the full-length receptor pool bound RAP (Figure 6C, lane 2) but in cultures subjected to brefeldin A, preventing access to TGN and cleavage, bindable receptors were not produced (lane 1).

The involvement of the furin consensus sequence site in the cleavage of prosortilin and the subsequent binding of ligand to sortilin finally was probed. Two mutant constructs of s-sortilin (Figure 1) were generated and expressed in CHO cells. In each mutant, two of the four residues R<sup>41</sup>WRR<sup>44</sup> constituting the consensus site for furin-mediated cleavage were substituted by either glycine or alanine, i.e. G<sup>41</sup>WRA<sup>44</sup> and R<sup>41</sup>WGG<sup>44</sup> (sm2- and sm1-sortilin, respectively). The constructs were expressed in CHO cells and, in accordance with the disruption of their furin target site sequence, both mutant proteins were secreted as uncleaved proforms, detectable with anti-propeptide antibody and with a slightly retarded electrophoretic migration compared with s-sortilin. Precipitation of the secreted biolabelled minireceptors by RAP beads confirmed the propeptide-mediated inhibition of ligand binding inasmuch as the mutant receptors, in particular sm2-sortilin, had a much lower affinity for RAP than did s-sortilin (Figure 7). As judged by densitometry, <3% of the mutant receptors were bound by RAP, in contrast to >90% of s-sortilin. It is noteworthy, however, that the sm1-sortilin persistently bound better to the RAP beads than did the sm2-mutant, suggesting that the propeptide cleavage site sequence might also be part of a structural element that conditions a close contact between the propeptide and its binding site in prosortilin.

The findings establish that the R<sup>41</sup>WRR<sup>44</sup> sequence is a target site for cleavage and that only converted sortilin exhibits significant RAP-binding activity.

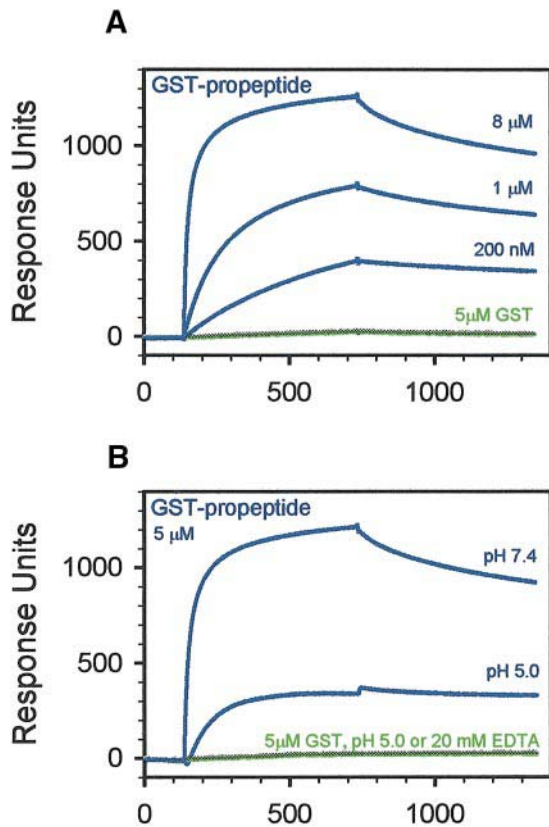
The question was then if cleavage *per se* was enough to induce binding activity. To test this, CHO transfectants were biolabelled and, after lysis in a calcium-free 1% Triton X-114 buffer at 4°C, the buffer phase was separated



**Fig. 8.** Induction of RAP binding by furin-mediated cleavage. Transfected CHO-K1 cells were labelled in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of brefeldin A and subsequently lysed in 1% Triton X-114 phosphate buffer containing EDTA. Following separation of the buffer phase from the detergent phase, the buffer phase was supplemented with either 5 mM EDTA (lanes 1 and 3) or with 2 mM Ca<sup>2+</sup> and furin (lanes 2 and 4). After 2 h at 37°C, and pre-clearance with blank beads, RAP-bindable sortilin was precipitated by incubation with RAP beads. Finally, the remaining unbound RAP was immunoprecipitated. An autoradiograph of a PPO-impregnated 8% gel is shown.

from the detergent. The buffer phase containing cellular s-sortilin was then supplemented with Ca<sup>2+</sup>, split in two and, prior to incubation, furin was added to only one of the samples. After 2 h at 37°C, sortilin was finally isolated using sequential precipitation with RAP beads and anti-sortilin beads. The outcome in terms of RAP binding and conversion is depicted in Figure 8. The figure shows that untreated cellular s-sortilin, in agreement with the above findings, constitutes uncleaved proreceptor with little or no binding activity (Figure 8, lanes 1 and 3, upper compared with lower panels). It further demonstrates that the situation changes completely upon incubation with furin. The s-sortilin has now been almost completely converted to its cleaved form (Figure 8, lane 1 and 3 compared with lanes 2 and 4, respectively), and the RAP-binding activity has increased many fold, as also evidenced by the depletion of sortilin left for immunoprecipitation.

It can be concluded that propeptide cleavage is necessary and sufficient for activation of RAP binding in sortilin. This is interesting since RAP is considered to bind and chaperone at least some of its target receptors in the early secretory pathway (Bu and Schwartz, 1998; Willnow, 1998). A similar role for RAP in the processing of sortilin seemed unlikely in view of the finding that newly synthesized sortilin is a proreceptor incapable of ligand binding. To clarify this, we examined and compared the sortilin expression in brain and testes of six normal and six RAP knockout mice and found that in terms of Western blotting and densitometry, the level of detectable sortilin per mg of tissue differed by <5% in the two groups.

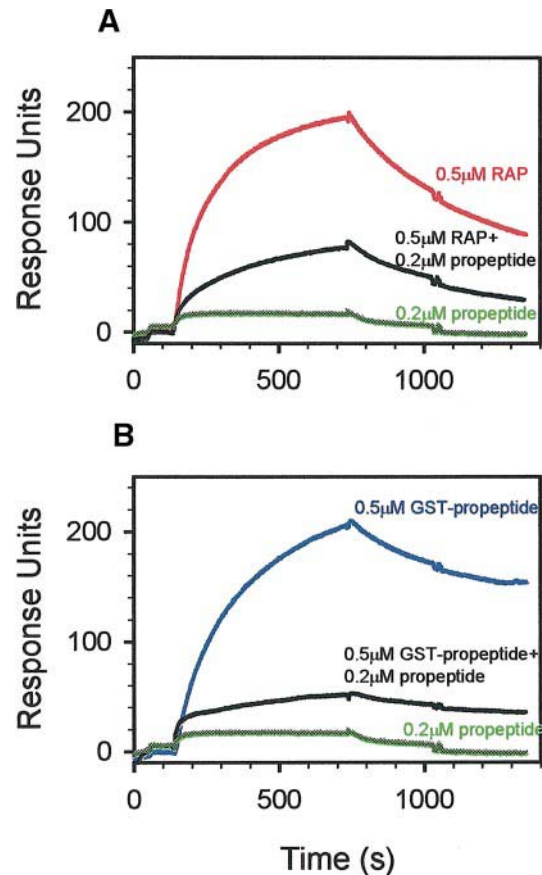


**Fig. 9.** Binding of GST-propeptide to purified s-sortilin. The GST-(sortilin)propeptide binding to sortilin was measured using surface plasmon resonance analysis. For screening, 50  $\mu\text{l}$  of a given sample were injected (5  $\mu\text{l}/\text{min}$ ) over flow cells carrying s-sortilin. (A) Surface plasmon resonance sensorgrams obtained at pH 7.4 with various concentrations of GST-propeptide and GST. (B) Surface plasmon resonance sensorgrams of 5  $\mu\text{M}$  GST-propeptide binding at pH 7.4 and 5, and the response obtained with 5  $\mu\text{M}$  GST at pH 5. All curves represent the response after subtraction of non-specific binding, which was determined as binding to a non-coated flow cell that had been activated and blocked.

Moreover, transfection of RAP knockout cells resulted in secretion of fully processed s-sortilin (not shown). These results suggest that RAP is not a molecular chaperone for sortilin.

#### **The sortilin propeptide inhibits binding of RAP and neurotensin**

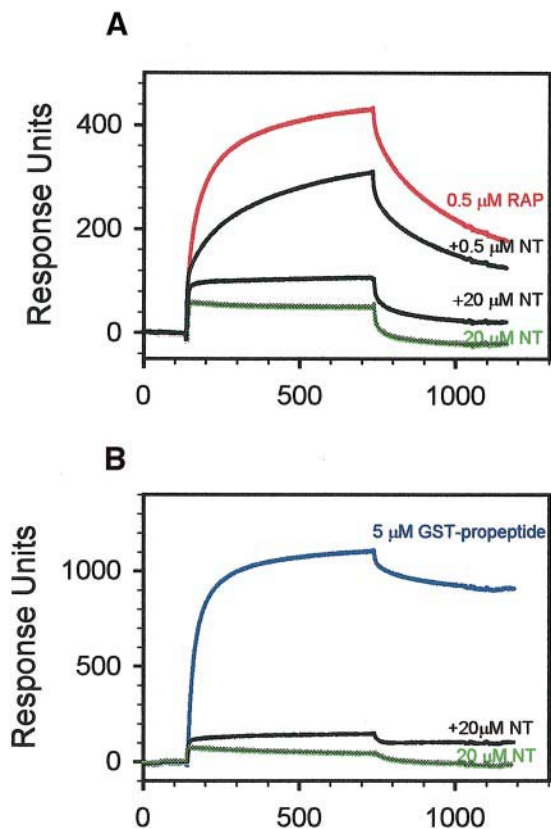
To understand why prosortilin binds ligand poorly, the sortilin propeptide was expressed as a GST fusion protein and its binding capacity was assayed by surface plasmon resonance using a sensor chip coated with RAP affinity-purified s-sortilin. As demonstrated in Figure 9A, the GST-propeptide fusion protein binds to s-sortilin, as opposed to control GST without propeptide. Binding is reversible, pH dependent (Figure 9B) and presents an estimated  $K_d$  in the region of 40 nM at pH 7.4, i.e. very similar to that previously determined for RAP. Experiments performed with free propeptide after removal of GST gave similar results, although propeptide on its own bound with a somewhat higher affinity ( $K_d \sim 20\text{--}30$  nM). Stoichiometric analysis performed at saturating ligand concentrations evidenced binding of  $\sim 0.5$  mol of GST-propeptide per mole of sortilin, indicating that sortilin interacts with its propeptide via a single site.



**Fig. 10.** Binding of RAP and GST-propeptide is inhibited by purified propeptide. Binding to immobilized sortilin was assayed by surface plasmon resonance as described in the legend to Figure 9. (A) Binding of 0.5  $\mu\text{M}$  RAP and of the sortilin propeptide (0.2  $\mu\text{M}$ ) released from the GST by thrombin-mediated cleavage of the fusion protein. The black sensorgram shows the surface plasmon resonance in response to combined binding of 0.5  $\mu\text{M}$  RAP and 0.2  $\mu\text{M}$  propeptide. (B) Binding of 0.5  $\mu\text{M}$  GST-propeptide and 0.2  $\mu\text{M}$  propeptide, and the response to their combined binding.

When GST-propeptide binding was probed under conditions that ensured saturation of all RAP-binding sites prior to the assay, no additional response was seen, suggesting that the two ligands were mutually inhibitive and bound to the same segment. This was explored further using propeptide (without GST) for inhibition of RAP. This is a more informative approach, since the propeptide, owing to its comparatively low molecular weight, induces a resonance response that differs significantly from that of RAP. Figure 10A shows that in terms of response units, the binding of 0.5  $\mu\text{M}$  RAP is easily distinguished from the binding of 0.2  $\mu\text{M}$  of propeptide. It follows that the intermediate response resulting from simultaneous binding at 0.5  $\mu\text{M}$  RAP and 0.2  $\mu\text{M}$  propeptide (Figure 10A) is a clear indication of a reduced contribution by RAP. GST-propeptide binding at 0.5  $\mu\text{M}$  was similarly affected by 0.2  $\mu\text{M}$  free propeptide (Figure 10B).

Sortilin recently has been identified as a receptor for the 13 amino acid neuropeptide neurotensin (Mazella *et al.*, 1998). Neurotensin bound to immobilized sortilin with high affinity (estimated  $K_d < 20$  nM) but, owing to its small size, the resonance response was low. However, its binding to sortilin is clearly demonstrated by its potent inhibition of the association between RAP and



**Fig. 11.** Neurotensin inhibits RAP and GST-propeptide binding to s-sortilin. The sensorgrams display the plasmon resonance response obtained from (A) RAP and (B) GST-propeptide binding at pH 7.4 to immobilized s-sortilin in unsupplemented buffer and in the presence of neurotensin (NT).

immobilized sortilin (Figure 11A). Thus, an equimolar amount of neurotensin produces a significant inhibition of RAP binding and, at a 40-fold excess of neurotensin, RAP binding is reduced to 10–20%. In control experiments, neurotensin did not inhibit RAP binding to the LRP, a RAP-binding member of the LDLR family, also suggesting that interaction between RAP and neurotensin did not account for the reduced RAP binding to sortilin in the presence of neurotensin. We finally examined if neurotensin interfered with the binding between immobilized sortilin and its propeptide, as would seem likely since neurotensin as well as the propeptide inhibits binding of RAP. The analysis of binding in the absence and presence of neurotensin is shown in Figure 11B. It can be deduced from the sensorgrams that 20 μM neurotensin almost completely abolished binding of 5 μM propeptide, and even a modest concentration of the neuropeptide caused a significant reduction in responsive units (not shown).

It can be concluded that the sortilin propeptide and the sortilin ligands neurotensin and RAP bind to the same or overlapping sites on the luminal domain of the fully processed receptor. The propeptide therefore appears to provide a steric hindrance, preventing the ligands from gaining access to the binding site in uncleaved prosortilin.

## Discussion

We show that sortilin is synthesized as a proreceptor which is converted to the mature ligand-binding receptor

by cleavage and release of a 44 amino acid N-terminal propeptide in the TGN or in post-TGN vesicles. Furin is abundant in these compartments and, since cleavage of prosortilin is pre-conditioned by a consensus sequence that is cleaved readily by furin *in vitro*, furin must be the main enzyme responsible for the processing. However, as the PCs of the subtilisin/Kex2-like family have overlapping substrate specificities, it is likely that other family members contribute to the conversion *in vivo*. Our findings further establish that the isolated propeptide binds to mature sortilin in a pH-sensitive manner and that it competes with external ligands for binding.

### **Sortilin is the first example of a receptor activated by furin cleavage**

Proteolytic cleavage is a common event in protein processing, and the Kex2-like PCs are important participants (Nakayama, 1997; Seidah and Chrétien, 1997). Along with PC 7, furin is the most widespread of the PCs and cleaves a large number of precursor proteins, including a few type-1 proreceptors. Furin itself is synthesized as a transmembrane proform and is converted to the active enzyme by self-mediated cleavage (Anderson *et al.*, 1997). As in sortilin, this results in release of an N-terminal propeptide and exposure of the active site. In contrast, the cleavage of sortilin and its functional implications differ markedly from cleavage of other proreceptors targeted by furin, e.g. the insulin and the insulin-like growth factor-1 receptors, the hepatocyte growth factor receptor and the LRP (Ulrich *et al.*, 1986; Mark *et al.*, 1992; Bravo *et al.*, 1994; Willnow *et al.*, 1996a). In these receptors, cleavage of the precursor proteins serves to generate the subunits that constitute the mature two-chain receptors and does not directly involve exposure of sites for ligand binding. Thus, the proforms of the insulin receptor, the hepatocyte growth factor receptor and the LRP are all capable of ligand binding, and major dysfunction of uncleaved receptors, as seen with the insulin-like growth factor-1 receptor, most likely results from a general malconformation (Mark *et al.*, 1992; Sugibayashi *et al.*, 1992; Komada *et al.*, 1993; Willnow *et al.*, 1996a; Lehmann *et al.*, 1998). Thus, sortilin is the first example of furin-mediated receptor activation. However, the mechanism may be shared by the receptors sorLA (Jacobsen *et al.*, 1996; Yamazaki *et al.*, 1996) and sorCS (Hermey *et al.*, 1998) which both have Vps10p domains containing furin consensus cleavage sites.

Activation of sortilin also has an outcome different from cleavage of mammalian proreceptors not targeted by furin. For instance, cleavage of the thyroid-stimulating hormone (TSH) proreceptor serves to generate the  $\alpha$ - and the  $\beta$ -chain of the mature receptor (Couet *et al.*, 1996). Other examples are the thrombin receptor and the proteinase-activated receptor-2 that are cleaved at the cell surface. These seven-transmembrane domain receptors each contain a concealed functional site which is exposed upon N-terminal cleavage of the proreceptors. However, unlike in sortilin, the functional sites do not bind external ligands but an intrinsic tethered ligand which irreversibly activates and downregulates the receptor (Vu *et al.*, 1991; Böhm *et al.*, 1996). It seems that the closest similarity to the mechanism of sortilin activation involves a 75 kDa type-1 plant membrane (PV75) recently isolated from

pumpkin. Like sortilin, PV75 contains a site which binds the propeptide and which is therefore inaccessible in the uncleaved proreceptor. Interestingly, PV75 and the related PV80 are both putative sorting receptors (Shimada *et al.*, 1997).

### **Putative importance of receptor activation in the TGN**

Our results suggest that the propeptide, while still attached to sortilin, can bind to a site downstream in the Vps10p domain and thereby prevent binding of other ligands. Alternatively, it cannot be excluded that the cleavage *per se* uncovers a concealed site for binding of isolated propeptide as well as external ligands. In either case, sortilin appears incapable of binding ligands to the Vps10p domain until it reaches compartments for furin-mediated cleavage and subsequent (acid-assisted) dissociation and clearance of propeptide. What is the reason for this inhibitory device in the early secretory pathway?

One possibility is that sortilin is a multifunctional receptor like members of the LDLR family. In fact, we have data to support this hypothesis since lipoprotein lipase binds to active sortilin with an affinity similar to its affinity for binding to LRP (M.S.Nielsen *et al.*, in preparation). It is conceivable that the sortilin propeptide functions to prohibit receptor aggregation provoked by premature binding of ligands in the ER and early Golgi. In the case of the multifunctional receptors gp330/megalin and LRP, which have no propeptide, this type of protection is provided by the ER-resident protein RAP, and RAP knockout seriously reduces LRP expression (Willnow *et al.*, 1995, 1996b). Thus, the sortilin propeptide and RAP may serve the same purpose, and our finding that RAP and the propeptide cross-compete for binding to sortilin might reflect a common theme in their recognition of the receptor. On the other hand, it is unlikely that RAP would be of any physiological importance to sortilin since it does not reach the TGN and, therefore, is unlikely to meet activated sortilin. The notion that RAP does not chaperone sortilin is in agreement with the finding that sortilin expression remains unchanged in RAP knockout mice.

Once activated, sortilin may contact its ligands in the TGN, in secretory vesicles or on the plasma membrane. It is interesting that sortilin is co-localized with CI-M6PR, presumably due to the homologous segments in their cytoplasmic tails (Petersen *et al.*, 1997). In adipocytes, this includes co-localization in vesicles containing the glucose transporter Glut4 as well as insulin-induced recruitment of the two receptors to the plasma membrane (Morris *et al.*, 1998). This suggests that sortilin, like CI-M6PR, may function both as a sorting receptor in the TGN, where most of the receptors are located, and as an endocytic receptor on the plasma membrane. Within this framework, activation of sortilin at the sorting starting point seems logical, whereas the CI-M6PR needs no activation since its mannose 6-phosphate-tagged ligands are unavailable till late in the synthetic pathway.

At present we have no evidence for sortilin-mediated sorting events in the TGN. Neurotensin so far is the only established sortilin ligand, and its generation from the neurotensin precursor depends on PCs that are active mainly in post-TGN vesicles (Barbero *et al.*, 1998). Further

studies should show whether sortilin is present in those vesicles. It will also be interesting to see if sortilin binds the neurotensin precursor and provides its trafficking to the secretory vesicles. The surface expression of sortilin is basically low and, once secreted, neurotensin binds mainly to the G-protein-coupled neurotensin receptors NT1 and NT2. However, following neurotensin-mediated down-regulation of the NT1 and NT2 receptors, sortilin is translocated to the cell surface where it most likely scavenges neurotensin, as suggested by Mazella *et al.* (1998). In all probability, uptake and degradation mediated by sortilin are part of the mechanism that terminates neurotensin signalling.

In conclusion, sortilin is activated by cleavage in the TGN and by the ensuing release of a propeptide that prevents premature binding of ligands to the receptor, a mechanism that may serve to protect the proreceptor during processing in the early secretory pathway.

## **Materials and methods**

### **RAP, antibodies and Western blotting**

Recombinant RAP was expressed in *Escherichia coli* and coupled to CNBr-activated Sepharose 4B (Pharmacia) as previously described (Petersen *et al.*, 1996). The soluble luminal domain of sortilin expressed in CHO-K1 cells (see below) was purified by RAP affinity chromatography (Tauris *et al.*, 1998) and used for generation of an anti-sortilin antibody. The Ig fraction was purified from rabbit serum using protein A-Sepharose and kept at a stock concentration of ~4–5 mg/ml. Antibodies recognizing the sortilin propeptide were generated (Neosystem, Strasbourg, France) using a synthetic peptide (PRWSGPIGVSWGLR) based on a sequence (residues P<sup>15</sup>–R<sup>28</sup>) in the N-terminus of uncleaved sortilin. Immunoblotting was performed as described elsewhere (Petersen *et al.*, 1997). The RAP knockout mice and fibroblasts have been described by Willnow *et al.* (1996b). To obtain extracts from mouse tissues, tissue samples were homogenized at 4°C in a 1% Triton X-100 buffer containing 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µg/ml Pefabloc (Boehringer, Mannheim, Germany) and, prior to Western blotting, the total protein concentration was determined using a kit from Sigma (BCA-1, St Louis, MO).

### **Constructs for eukaryotic expression**

The fl-sortilin construct was generated by inserting an *Xba*I–*Sma*I fragment (Figure 1) from the original library vector (pBK-CMV; Stratagene, La Jolla, CA) into the pCDNA3.1/Zeo(–) vector from Invitrogen (Carlsbad, CA). The construct encoding the soluble mini-receptor (s-sortilin) comprising the N-terminal luminal domain (residues M<sup>–33</sup>–S<sup>725</sup>), and including a C-terminal His<sub>6</sub> tag, was expressed in the pCDNA3.1/Zeo(–) vector by sequentially inserting C- and N-terminal cDNA fragments separated by the *Bsp*EI restriction site at position 2265. The C-terminal domain was PCR-amplified using primers S1 and S2 followed by digestion with *Xho*I and *Not*I. The resulting fragment was inserted into the pCDNA3.1/Zeo(–) vector. The N-terminal *Xba*I–*Bsp*EI fragment was then inserted into the *Xba*I–*Bsp*EI-digested vector containing the C-terminal His<sub>6</sub>-tagged fragment.

Soluble mutant minireceptors were generated by altering the furin cleavage site R<sup>41</sup>WRR<sup>44</sup> to either R<sup>41</sup>WGG<sup>44</sup> (sm1-sortilin) or A<sup>41</sup>WRG<sup>44</sup> (sm2-sortilin) using a two-step PCR procedure (Ho *et al.*, 1989). The high content of GC bases at the 5' end of the sortilin cDNA rendered conventional PCR impossible. The problem was circumvented by cloning the *Not*I–*Hind*III fragment spanning positions 206–903 into the pBluescript vector and using a PCR kit (Advantage-GC) from Clontech (Palo Alto, CA) designed for amplifying DNA with high GC content. Overlapping 5' and 3' PCR fragments were amplified from the pBluescript construct, using the mutated and appropriate flanking primers (see below). The primary fragments were separated in low melting agarose and PCR ligated, using the flanking primers. The resulting PCR fragments were digested with *Not*I and *Hpa*I, and the wild-type and the mutated fragments were exchanged in the pCDNA3.1/Zeo(–) vector carrying the fl-sortilin cDNA and sequenced to confirm the mutations. The 5'-mutated *Xba*I–*Hpa*I fragments from the full-length constructs finally were transferred to the soluble sortilin construct. Figure 1 shows



a schematic presentation of the expression products and a map of restriction sites in the sortilin cDNA. Primers used were: for the s-sortilin construct, CGCCTCGAGCTGGCACACCCACAGACC (2101–2116, S1) and GCGCGGCCGGCCCTAATGATGATGATGATGATGAG-AATTTGACTTGGAAAT (2279–2295, S2); for sm1-sortilin, CGTTGG-GGTGGCAGCGC (241–257, 5' fragment) and GCGCTGCCACCC-CAACG (241–257, 3' fragment); for sm2-sortilin, GCTGCCACGCCA-AGCGCCG (236–255, 5' fragment) and GCTTGGCGTGGCAGCGC (241–257, 3' fragment); and flanking primers, TTCGAAAATCTGATG-ATCTG (616–635, downstream) and CCAAGCTCGAAATTA CCCT-CACT (upstream pBluescript).

### Cells and transfection

All transfections were carried out using CHO-K1 cells and RAP knockout mouse fibroblasts kindly provided by Dr T. Willnow (Max-Delbrück-Center for Molecular Medicine, Berlin). CHO cells were cultured in serum-free HyQ-CCM5 CHO medium (HyClone, Logan, UT), and the mouse fibroblasts in minimum essential medium with Ultraglutamine and 10% fetal calf serum (FCS).

The pcDNA/Zeo(-) constructs, containing full-length sortilin and soluble minireceptors, were transfected into CHO cells and RAP knockout cells using DC-Chol (Gao and Huang, 1991). For selection of stable transfectants, the cells were cultured in medium containing 500 µg zeocin per ml (Invitrogen, Faraday, CA) and cloned. Positive clones were identified by immunocytochemistry and/or by Western blotting performed on culture medium and/or cell lysates.

### Propeptide expression

For *E. coli* expression, the sortilin propeptide (residues Q<sup>1</sup>–R<sup>44</sup>) was PCR-amplified using an upstream primer including a factor X consensus cleavage sequence. Following *Bam*HI and *Hind*III digestion, the resulting fragment was cloned and inserted into the pT7-PL vector (Christensen *et al.*, 1991) and expressed as a N-terminal His<sub>6</sub> fusion protein. However, as yields were low, the *Bam*HI–*Eco*RI fragment encoding the propeptide was moved from pT7-PL into the pGEX4T-1 vector (Pharmacia) containing a thrombin cleavage site. The primers used were: CACGGAT-CCATCGAGGGTAGGCAGGACCGG (121–134, 5') and CACAAG-CTTAGCGACGCCAACG (241–252, 3'). The construct was expressed in bacterial strain BL21(DE3) and purified by affinity chromatography on a glutathione-agarose column (Pharmacia). The fusion product was cleaved with thrombin (Sigma) and the released propeptide was separated from GST and residual fusion product using centricon-10 (Amicon, Beverly, MA) and concentrated using centricon-3.

### Metabolic labelling and affinity precipitation

Transfected cells, expressing various constructs of sortilin, were cultured in Nunclone Multidishes (Nunc, Roskilde, Denmark). At ~80% confluency, the cells were washed twice in Tris/balanced salt solution pH 7.5 (BSS) and incubated for 10 min in modified Eagle's medium without cysteine and methionine (Sigma Chemical Co., St Louis, MO) before biolabelling in the same medium (200–500 µl/well). Labelling was performed in the absence or presence of 10 µg/ml brefeldin A (Sigma) using 200 µCi of L-[<sup>35</sup>S]cysteine and L-[<sup>35</sup>S]methionine per ml of medium (Pro-mix; Amersham International, Buckinghamshire, UK). At the end of the labelling period (1–3 h), the medium was harvested and the cells were washed twice with BSS (4°C) and subsequently lysed for 10 min at 4°C in 1% Triton X-100 (20 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, pH 8.0) supplemented with a proteinase inhibitor cocktail (CompleteMini; Boehringer, Mannheim, Germany). Alternatively, for chase experiments, the labelled cells were washed twice in BSS and reincubated in unsupplemented 15% FCS full medium for 0–3 h before lysis. Cell lysates (200 µl) were supplemented with 600 µl of BSS and 200 µl of medium, while 600 µl of BSS and 200 µl of lysis buffer were added to the medium, prior to addition of 2.5 µl of anti-sortilin Ig and incubation (4°C, 1 h). Precipitation was completed by addition of 50 µl of GammaBind G-Sepharose beads (Pharmacia). The beads were washed five times in 1 ml of 0.05% Tween-20 BSS and either boiled for 2 min in 100 µl of reducing sample buffer [10 mM dithiothreitol (DTT), 2.5% SDS] or resuspended for deglycosylation as described below.

For RAP binding, the cell lysate and the medium were diluted as above and pre-incubated (4°C, 2 h) with 75 µl of uncoupled CNBr-activated Sepharose 4B beads (Pharmacia) prior to incubation (4°C, 6 h) with RAP-Sepharose beads coupled with recombinant RAP. The beads were washed five times in 0.05% Tween-20 before resuspension in reducing SDS sample buffer. Finally, unbound sortilin was precipitated from the supernatant using anti-sortilin and GammaBind G beads as described above. The precipitated proteins were analysed by reducing

SDS-PAGE, and diphenyloxazole-fluorographed gels were exposed at –70°C.

### Furin-mediated cleavage

*In vitro* translation of soluble sortilin, using a kit from Promega (Madison, WI), was performed for 30 min at 30°C in a reaction mixture containing 5 µl of erythrocyte lysate, 0.4 µl of reaction buffer, 0.25 µl of RNA guard, 0.8 µl of Pro-mix, 0.25 µl of T7 polymerase, 1.5 µl (0.25 mg/ml) of DNA and 0.55 µl of water. Two µl of diluted reaction mixture (1:5 in phosphate-buffered saline; PBS) were supplemented with 2 µl of PBS, 0.2 µl of 10 mM CaCl<sub>2</sub>, 0.2 µl of 2 mM DTT and 1 µl (~1 U) of recombinant furin (Alexis, San Diego, CA) and incubated at 37°C for 1 h. The reaction was stopped by addition of reducing SDS sample buffer, and aliquots were analysed by SDS-PAGE.

For cleavage of cellular sortilin, CHO-K1 cells expressing s-sortilin were biolabelled as described above. The cells were then washed in BSS and lysed in 1% Triton X-114 phosphate buffer pH 7.4 (5 min at 4°C) and, after heating the lysate (30°C), the buffer and the detergent phases were separated by centrifugation. The buffer phase (150 µl), containing s-sortilin, was supplemented with 30 µl of 20 mM EDTA, or a similar volume of 10 mM Ca<sup>2+</sup> containing ~10 U of furin, and incubated for 2 h at 37°C. Following incubation, medium and Triton X-100 lysis buffer were added to a final volume of 800 µl (0.1% detergent) and s-sortilin was either immunoprecipitated or precipitated with RAP-Sepharose beads as described above.

### Analysis of glycosylation

Biolabelled sortilin, full-length receptor or s-sortilin was precipitated from cell lysates or medium using anti-sortilin Ig and GammaBind G beads. For treatment with PNGase-F, washed beads were heated (95°C, 3 min) in 10 µl of 1% SDS, heated again after addition of 90 µl of 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM EDTA, 10 mM Na-azide, 0.5% Triton X-100 pH 7.2, and finally cooled before addition of 0.5 U of PNGase-F. Alternatively, beads were resuspended in 20 mM Na-citrate, 2 mM CaCl<sub>2</sub>, 150 mM NaCl, pH 6.0 containing 0.01 U of neuraminidase, or in 50 mM phosphate, 0.01% SDS, 1 M mercaptoethanol, pH 5.5 containing 4 mU of endoglycosidase H (Endo-H). After overnight incubation at 30°C, reactions were stopped by addition of an equal volume of reducing SDS sample buffer, and the samples were analysed by SDS-PAGE and autoradiography. All glycosidases were from Boehringer.

### Biosensor measurements

The neuropeptide neurotensin (N6383) was from Sigma. All measurements were performed on a BIAcore 2000 instrument (Biacore Sweden) equipped with CM5 sensor chips maintained at 20°C. A continuous flow of HBS buffer (10 mM HEPES pH 7.4, 3.4 mM EDTA, 150 mM NaCl, 0.005% surfactant P20) passing over the sensor surface was maintained at 5 µl/min. The carboxylated dextran matrix of the sensor chip flow cells 1–3 was activated by the injection of a solution containing 0.2 M *N*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide and 0.05 M *N*-hydroxy-succinimide in water. A sortilin solution (320 µl, 5 µg/ml in 10 mM sodium acetate pH 4.0) was then injected over flow cells 1 and 2 at a flow rate of 15 µl/min. Remaining binding sites in all three flow cells were blocked by injection (5 µl/min) of 70 µl of 1 M ethanolamine pH 8.5. The surface plasmon resonance signal from immobilized sortilin generated 4419 and 6166 BIAcore response units (RU) equivalent to 49 and 69 fmol/mm<sup>2</sup>.

Screening of the samples was performed by injecting aliquots of 50 µl, at concentrations of 0.1–8 µM, through all flow cells with a flow rate of 5 µl/min. Unless otherwise stated, the samples were dissolved in 10 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM CaCl<sub>2</sub>, 1 mM EGTA, 0.005% surfactant P20. Sample buffer was also used as running buffer. The BIAcore response is expressed in relative response units (RU), i.e. the difference in response between the immobilized protein flow cell and the corresponding control flow cell (activated and blocked but without protein). Regeneration of the sensor chip after each cycle of analysis was performed by injecting 20 µl of 10 mM glycine/HCl pH 4.0, 500 mM NaCl, 20 mM EDTA and 0.005% surfactant P20. For calcium-free conditions, HBS containing 20 mM EDTA was used as sample as well as running buffer. Kinetic parameters were determined by using the BIAevaluation 3.0 software.

### Acknowledgements

Nina Jørgensen is thanked for skilled technical assistance. The study was supported by the Danish Medical Research Foundation, Carlsberg Foundation and the John and Birthe Meyer Foundation.

## References

- Anderson, E.D., VanSlyke, J.K., Thulin, C.D., Jean, F. and Thomas, G. (1997) Activation of the furin endoprotease is a multiple-step process: requirements for acidification and internal propeptide cleavage. *EMBO J.*, **16**, 1508–1518.
- Barbero, P., Rovere, C., De Bie, I., Seidah, N., Beaudet, A. and Kitabgi, P. (1998) PC5-A mediated processing of pro-neurotensin in early compartments of the regulated secretory pathway of PC5-transfected PC12 cells. *J. Biol. Chem.*, **273**, 25339–25346.
- Bean, A.J., Dagerlind, A., Hokfelt, T. and Dobner, P.R. (1992) Cloning of human neurotensin/neuromedin N genomic sequences and expression in the ventral mesencephalon of schizophrenics and age/sex matched controls. *Neuroscience*, **50**, 259–268.
- Bohm, S.K., Khitin, L.M., Grady, E.F., Aponte, G., Payan, D.G. and Bunnett, N.W. (1996) Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. *J. Biol. Chem.*, **271**, 22003–22016.
- Bravo, D.A., Gleason, J.B., Sanchez, R.I., Roth, R.A. and Fullar, R.S. (1994) Accurate and efficient cleavage of the human insulin proreceptor by the human proprotein-processing protease furin. *J. Biol. Chem.*, **269**, 25830–25837.
- Bu, G. and Schwartz, A.L. (1998) RAP, a novel type of ER chaperone. *Trends Cell. Biol.*, **8**, 272–276.
- Chabry, J., Gaudriault, G., Vincent, J.P. and Mazella, J.P. (1993) Implication of various forms of neurotensin receptors in the mechanism of internalization of neurotensin in cerebral neurons. *J. Biol. Chem.*, **268**, 17138–17144.
- Chalon, P., Vita, N., Kaghad, M., Guillemont, M., Bonnin, J., Delpech, B., Le Fur, G., Ferrara, P. and Caput, D. (1996) Molecular cloning of a levocabastine-sensitive neurotensin binding site. *FEBS Lett.*, **386**, 91–94.
- Christensen, J.H., Hansen, P.K., Lillelund, O. and Thøgersen, H.C. (1991) Sequence-specific binding of the three-finger fragment *Xenopus* transcription factor IIIA to the internal control region of a 5S RNA gene. *FEBS Lett.*, **281**, 181–184.
- Couet, J., de Bernard, S., Loosfelt, H., Saunier, B., Milgrom, E. and Misraki, M. (1996) Cell surface protein disulfide-isomerase is involved in the shedding of human thyrotropin receptor ectodomain. *Biochemistry*, **35**, 14800–14805.
- Dobner, P.R., Barber, D.L., Villa-Komaroff, L., McKiernan, C. (1987) Cloning and sequence analysis of cDNA for the canine neurotensin/neuromedin N precursor. *Proc. Natl Acad. Sci. USA*, **84**, 3516–3520.
- Gao, X. and Huang, L. (1991) A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem. Biophys. Res. Commun.*, **179**, 280–285.
- Gliemann, J. (1998) Receptors of the low density lipoprotein (LDL) receptor family in man. Multiple functions of the large family members via interaction with complex ligands. *Biol. Chem.*, **379**, 951–964.
- Hermey, G., Hermans-Borgmeyer, I. and Schaller, H.C. (1998) SorCS a novel neurospecifically expressed gene with homology to sorting receptors. *Biol. Chem. Suppl.*, **379**, 77.
- Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene*, **77**, 51–59.
- Jacobsen, L., Madsen, P., Moestrup, S.K., Lund, A.H., Tommerup, N., Nykjær, A., Sottrup-Jensen, L., Gliemann, J. and Petersen, C.M. (1996) Molecular characterization of a novel human hybrid-type receptor that binds the  $\alpha 2$ -macroglobulin receptor-associated protein. *J. Biol. Chem.*, **271**, 31379–31383.
- Komada, M., Hatsuzawa, K., Shibamoto, S., Ito, F., Nakayama, K. and Kitamura, N. (1993) Proteolytic processing of the hepatocyte growth factor/scatter factor receptor by furin. *FEBS Lett.*, **328**, 25–29.
- Lehmann, M., Andre, F., Bellan, C., Remacle-Bonnet, M., Garouste, F., Parat, F., Lissitsky, J.C., Marvaldi, J. and Pommier, G. (1998) Deficient processing and activity of type I insulin-like growth factor receptor in the furin-deficient LoVo-C5 cells. *Endocrinology*, **139**, 3763–3771.
- Lin, B.-Z., Pilch, P.F. and Kandror, K.V. (1997) Sortilin is a major protein component of glut4-containing vesicles. *J. Biol. Chem.*, **272**, 24145–24147.
- Marcusson, E.G., Horazdovsky, B.F., Cereghino, J.L., Gharakhanian, E. and Emr, S.D. (1994) The sorting receptor for yeast vacuolar carboxypeptidase Y is encoded by the *VPS10* gene. *Cell*, **77**, 579–586.
- Mark, M.R., Lokker, N.A., Zioncheck, T.F., Luis, E.A. and Godowski, P.J. (1992) Expression and characterization of hepatocyte growth factor receptor-IgG fusion proteins. Effects of mutations in the potential proteolytic cleavage site on processing and ligand binding. *J. Biol. Chem.*, **267**, 26166–26171.
- Mazella, J. *et al.* (1998) The 100-kDa neurotensin receptor is gp95/sortilin, a non-G-protein-coupled receptor. *J. Biol. Chem.*, **273**, 26273–26276.
- Misumi, Y., Misumi, Y., Miki, K., Takatsuki, A., Tamura, G. and Ikehara, Y. (1986) Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J. Biol. Chem.*, **261**, 11398–11403.
- Morris, N.J., Ross, S.A., Lane, W.S., Moestrup, S.M., Petersen, C.M., Keller, S.R. and Lienhard, G.E. (1998) Sortilin is the major 110-kDa protein in glut4 vesicles from adipocytes. *J. Biol. Chem.*, **273**, 3582–3587.
- Nakayama, K. (1997) Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. *Biochem J.*, **327**, 625–635.
- Obermoeller, L.M., Warshawsky, I., Wardell, M.R. and Bu, G. (1997) Differential functions of triplicated repeats suggest two independent roles for the receptor-associated protein as a molecular chaperone. *J. Biol. Chem.*, **272**, 10761–10768.
- Petersen, C.M. *et al.* (1996) The receptor-associated protein (RAP) binds calmodulin and is phosphorylated by calmodulin-dependent kinase II. *EMBO J.*, **15**, 4165–4178.
- Petersen, C.M. *et al.* (1997) Molecular identification of a novel candidate sorting receptor purified from human brain by receptor-associated protein affinity chromatography. *J. Biol. Chem.*, **272**, 3599–3605.
- Seidah, N.G. and Chrétien, M. (1997) Eukaryotic protein processing: endoproteolysis of precursor proteins. *Curr. Opin. Biotechnol.*, **8**, 602–607.
- Shimada, T., Kuroyanagi, M., Nishimura, M. and Hura-Nishimura, I. (1997) A pumpkin 72-kDa membrane receptor protein of precursor-accumulating vesicles has characteristics of a vacuolar sorting receptor. *Plant Cell Physiol.*, **38**, 1414–1420.
- Sugibayashi, M., Shigeta, Y., Teraoka, H. and Kobayashi, M. (1992) Characterization of unprocessed insulin proreceptors in Cos7 cells transfected with cDNA with arg735-ser735 point mutation at the cleavage site. *Metabolism*, **41**, 820–826.
- Tanaka, K., Masu, M. and Nakanishi, S. (1990) Structure and functional expression of the cloned rat neurotensin receptor. *Neuron*, **6**, 847–854.
- Tauris, J., Ellgaard, L., Jacobsen, C., Nielsen, M.S., Madsen, P., Thøgersen, H.C., Gliemann, J., Petersen, C.M. and Moestrup, S.K. (1998) The carboxy-terminal domain of the receptor-associated protein binds to the Vps10 domain of sortilin. *FEBS Lett.*, **429**, 27–30.
- Ullrich, A. *et al.* (1986) Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J.*, **5**, 2503–2512.
- Vincent, J.-P. (1995) Neurotensin receptors: binding properties, transduction pathways, and structure. *Cell. Mol. Neurobiol.*, **15**, 501–512.
- Vu, T.-K.H., Hung, D.T., Wheaton, V.I. and Coughlin, S.R. (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell*, **64**, 1057–1068.
- Willnow, T. (1998) Receptor-associated protein (RAP): a specialized chaperone for endocytic receptors. *Biol. Chem.*, **379**, 1025–1031.
- Willnow, T.E., Armstrong, S.A., Hammer, R.E. and Herz, J. (1995) Functional expression of low density lipoprotein receptor-related protein is controlled by receptor-associated protein *in vivo*. *Proc. Natl Acad. Sci. USA*, **92**, 4537–4531.
- Willnow, T., Moehring, J.M., Inocencio, N.M., Moehring, T.J. and Herz, J. (1996a) The low-density-lipoprotein receptor-related protein (LRP) is processed by furin *in vivo* and *in vitro*. *Biochem. J.*, **313**, 71–76.
- Willnow, T.E., Rohlmann, A., Horton, J., Otani, H., Braun, J.R., Hammer, R.E. and Herz, J. (1996b) RAP, a specialized chaperone, prevents ligand-induced ER retention and degradation of LDL receptor-related endocytic receptors. *EMBO J.*, **15**, 2632–2639.
- Yamazaki, H., Bujo, H., Kusunoki, J., Seimiya, K., Kanaki, T., Morisaki, N., Schneider, W.J. and Saito, Y. (1996) Elements of neural adhesion molecules and a yeast vacuolar protein sorting receptor are present in a novel mammalian low density lipoprotein receptor family member. *J. Biol. Chem.*, **271**, 24761–24768.

Received November 4, 1998; revised December 2, 1998;  
accepted December 3, 1998