Identification of a Transferable Sorting Domain for the Regulated Pathway in the Prohormone Convertase PC2*

(Received for publication, May 23, 1996, and in revised form, July 15, 1996)

John W. M. Creemers‡§§, Elena F. Usac‡§§, Nicholas A. Bright‡, Jan-Willem Van de Loo**, Erik Jansen**, Wim J. M. Van de Ven**, and John C. Hutton‡‡‡

From the ‡Department of Clinical Biochemistry, University of Cambridge, Addenbrookes Hospital, Hills Road, Cambridge CB2 2QR, United Kingdom and the **Laboratory for Molecular Oncology, Center for Human Genetics, University of Leuven and Flanders Interuniversity Institute for Biotechnology, Herestraat 49, B-3000 Leuven, Belgium

The mammalian subtilisin-like endoproteases furin and PC2 catalyze similar reactions but in different parts of the cell: furin in the trans-Golgi network and PC2 in dense-core granules. To map targeting domains within PC2, chimeras were constructed of the pro-, catalytic, and middle domains of furin with the carboxyl-terminal domain of PC2 (F-S-P) or of the pro- and catalytic domains of furin with the middle and carboxyl-terminal domains of PC2 (F-N-P). Their behavior in stable transfected AtT-20 cells was compared to a furin mutant truncated after the middle domain (F-S), wild-type furin, and with wild-type PC2. F-S-P, F-N-P, and F-S were catalytically active and underwent post-translational proteolysis and N-glycosylation with similar kinetics to wild-type furin. The truncated furin mutant was not stored intracellularly, whereas both chimeras, like PC2, showed intracellular retention and regulated release. Immunofluorescence and immuno-electron microscopy showed the presence of the chimeras and PC2 in dense-core secretory granules together with proopiomelanocortin immunoreactivity. PC2 was sorted more efficiently than F-S-P, and the inclusion of the middle domain (F-N-P) further enhanced intracellular retention. It is concluded that sorting of PC2 into the regulated pathway depends on its carboxyl terminus. The middle domain may provide additional sorting determinants or a conformational framework for expression of the sorting signal.

The majority of eukaryotic secretory proteins are co-translationally translocated into the lumen of the endoplasmic reticulum (ER) and, upon reaching the TGN, are either targeted to dense-core secretory granules for storage or released constitutively by a default mechanism (Moore and Kelly, 1986). Active sorting to the granule is postulated to occur either by a receptor-mediated process (Chung et al., 1989) or be a consequence of aggregation promoted by the lowering of pH and elevation of Ca²⁺ in the TGN (Kelly, 1985; Chanat and Huttner, 1991).

One approach to the identification of sorting sequences or domains in secreted proteins has been to study the effects of deletion or mutation of candidate sequences within granule proteins and investigation of whether transfer of such sequences to constitutively secreted proteins reroutes them to the granules (Moore and Kelly, 1986). A major limitation of this approach, particularly the case for gene transfer experiments performed with chimeras of heterologous proteins, is that any perturbation of the three-dimensional structure of the protein is likely to mask the activity of a putative sorting motif. To minimize these effects, we have chosen to perform experiments with the family of mammalian subtilisin-like proprotein processing enzymes (reviewed by Hutton, 1990; Steiner et al., 1992; Van de Ven et al., 1993; Halban and Irminger, 1994; Seidah et al., 1994), which includes proteins, like PC1 and PC2, that are sorted into secretory granules and TGN-resident proteins such as furin. The seven family members currently documented all exhibit a common domain structure characterized by a short unique amino-terminal signal peptide, a moderately conserved pro-domain usually of 70–90 aa terminating in an endoproteolytic cleavage site comprised of a cluster of basic residues, a highly conserved subtilisin-like catalytic domain of 320–340 aa, and a well conserved “middle” domain of 110–130 aa followed by unique carboxyl-terminal domains varying from 50 to several hundred residues in length. The domain boundaries are readily recognizable on the basis of homology and are amenable to domain-swapping experiments.

In this study, we have chosen prohormone convertase PC2 (Seidah et al., 1990; Smeekens and Steiner, 1990) as a model enzyme for the regulated pathway and furin (Roebroek et al., 1986; Van de Ven et al., 1990) as the partner for the domain-swapping experiments and focused on the middle and carboxy-terminal domains as probable sites of localization of a targeting signal. The 50-aa carboxyl terminus of PC2 terminates in a putative amphipathic α-helix and is preceded by a stretch of charged aa, a feature which is also apparent in PC1 (Smeekens et al. 1991) and the unrelated secretory granule metalloexopeptidase carboxypeptidase E (H) (Parkinson, 1990; Mitra et al. 1994). The 232-aa carboxyl terminus of furin is comprised of a cysteine-rich region, a membrane-spanning segment, and a hormone convertase 2; POMC, proopiomelanocortin; TGN, trans-Golgi network; vWF, von Willebrand factor; PBS, phosphate-buffered saline; CPH, carboxypeptidase H (or E); aa, amino acid(s); PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.
58-aa cytoplasmic tail. Previous studies with furin have shown that the catalytic and middle domains are sufficient for catalytic activity (Hatsuzawa et al., 1992; Creemers et al., 1993), while the transmembrane domain and the cytoplasmic tail are essential for localization in the TGN (Bosshart et al., 1994; Molloy et al., 1994; Schäfer et al., 1995). The function of the cysteine-rich region (Roebroek et al., 1992) is unclear.

Our results indicate that the domain-swapping strategy was successful in that normal post-translational processing and catalytic activity of the constructs was achieved. The experiments indicate that a targeting motif is located in the carboxyl terminus of PC2, but efficient sorting may also depend upon an extended structure incorporating the middle domain of the protein.

MATERIALS AND METHODS

Construction of Mutants and Chimeric Proteins of Furin and PC2—A 4.1-kilobase human FUR-cDNA (Van den Ouweland et al., 1990) and a 2.2-kilobase human PC2-cDNA (provided by Dr. D. Steiner, Chicago; Smeekens and Steiner, 1990), containing all coding sequences, were cloned into the EcoRI site of pSELECT (Promega, Southampton, United Kingdom). NarI and SpeI restriction sites were introduced using the altered sites in vitro mutagenesis system (Promega) according to the guidelines of the supplier. The sequence of the mutagenic primers used to generate the SpeI site in the FUR-cDNA was 5’-GACCAAGGTTCAC-A’CTA*GTACTCTATGGC-3’ (the asterisks indicate the mutations; see also Fig. 1). A NarI site at the junction of the catalytic and middle domains occurs in native FUR-cDNA. The sequences of the primers used to generate a NarI and a SpeI site in the PC2-cDNA were 5’-CC-TTGATGCAAGGCTCAGTGGAAAG-3’ and 5’-GAAAGGACTGGACAGCTAGGA-3’G*ATGCTCTATTGGCA-3’. These restriction sites were then used in combination with restriction sites in the vector to generate the cDNAs encoding the proteins (see Fig. 1). The truncated form of furin was constructed by ligating the 1.8-kilobase EcoRI-SpeI 5’-end fragment of the FUR mutant into pEGEM357(+)’, digested with EcoRI and BamHI, together with a complementary primer set with a 5’-SpeI and a 3’-BamHI overhang (forward primer, 5’-CTA-GTACTCTATTGGCAATCT-3’; reverse primer, 5’-GATCCTAGGTGC-GTG*TGCTGTATGCA-3’). This primer set contains the sequences of the FUR cDNA between the SpeI site and the codon for Thr572 and introduces an amber stop codon after Thr572. All mutations were confirmed by nucleotide sequence analysis. The constructs were subcloned in the mammalian expression vector pcDNA3 (In Vitrogen Corp., Abingdon, UK).

Cell Culture and Stable Transfection of A7T20 Cells—All cell culture media were obtained from Sigma Chemicals (Poole, UK). The mouse pituitary tumor cell line A7T20 was grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics (penicillin (100 units/ml) and streptomycin (100 μg/ml)). PK15 cells (pig kidney cells) were maintained as described before (Creemers et al., 1993).

Transfections were performed using LipofectAMINE reagent (Life Technologies, Inc., Paisley, UK) according to the supplier’s recommendations. Briefly, cells grown to 80% confluency in a 3.5-cm well were incubated with a mixture of 1 μg of DNA and 12 μl of LipofectAMINE in a total volume of 1 ml of serum-free medium (OptiMEM; Life Technologies, Inc., Paisley, UK) according to the supplier’s recommendation. Briefly, cells grown to 80% confluency in a 3.5-cm well were incubated with a mixture of 1 μg of DNA and 12 μl of LipofectAMINE in a total volume of 1 ml of serum-free medium (OptiMEM; Life Technologies, Inc., Paisley, UK) according to the supplier’s recommendation.

Indirect Immunofluorescence—Cells grown on glass slides were rinsed twice in PBS and then fixed in 4% (w/v) paraformaldehyde followed by permeabilization with 0.05% saponin was performed as this provided the best contrast for these markers while the signal for the recombinant proteins was not affected. Experiments were performed at least twice. All incubation steps and washes were performed in PBS supplemented with 0.2% bovine serum albumin (BSA; Boehringer Mannheim), a rabbit anti-furin antibody MON-148 (Lys-Asp-Glu-Leu) antibody (provided by Dr. G. Butter, Cambridge, UK), a rabbit anti-TGN38 antibody (provided by Dr. G. Banting, Bristol, UK), a rabbit anti-proopiomelanocortin (POMC) antibody recognizing the amino-terminal propeptide of POFC (provided by Dr. P. J. Lowry, Reading, UK), and a mouse anti-POMC antibody (provided by Dr. A. White, Manchester, UK). For double labeling, fluorescein or Texas red-conjugated sheep anti-mouse Ig (Amersham, PLC, Amersham, UK), donkey anti-rabbit Ig (Amersham), and goat anti-rat Ig (Sigma) were used. Cells were examined with a Zeiss Axiosplan microscope equipped with UV optics.

Immunoelectron Microscopy—Cells (8–10 × 10⁶) were transfected as described above and incubated overnight in serum-free Dulbecco’s modified Eagle’s medium supplemented with 10 μM Rp-cAMP (Biolog, Bremen, Germany), a protein kinase A inhibitor. The next day, cells were cultured for 3 h in the presence of 10 μM forskolin (Sigma) and 0.1 mm IBMX (Sigma) or the carrier solvent (0.01% dimethyl sulfoxide). This protocol was developed to minimize the tonic release of stored material induced by serum factors while maximizing the response to elevations in intracellular cyclic AMP. Transiently transfected cells were used in the preference to stable lines to avoid potential artifact due to clonal variation. The released proteins were precipitated with ice-cold 5% (w/v) trichloroacetic acid and then solubilized in SDSPAGE sample buffer. Cells were lysed directly in sample buffer and run in parallel on 7.5% SDS-PAGE gels. The electrophoresed proteins were transferred to nitrocellulose and probed with either MON-148 or a rabbit anti-PC2 antisemur followed by a peroxidase-conjugated secondary antibody (Sero-tec) and ECL detection (Amersham). Quantitation was achieved using densitometry (Personal Densimeter, Molecular Dynamics).

Immunoelectron Microscopy—Cells were prepared for ultrastructural immunocytochemistry essentially as outlined by Griffiths (1993). Cells grown on culture slides were fixed with PBS and then washed with 250 mM HEPES, pH 7.2, containing 8% paraformaldehyde at room temperature for 1 h. The cells were then washed, permeabilized, and incubated in fresh fixative at 4 °C. The fixed cells were embedded in warm 10% gelatin, cooled, trimmed, and infused with 2.1M sucrose in PBS overnight at 4 °C and then frozen in liquid nitrogen. Frozen ultra-thin sections were cut using a Reichert Ultracut S cryoultramicrotome equipped with an FCS cryochamber attachment (Leica, Milton Keynes, UK). Tissue sections were collected in PBS containing 2.3 mM sucrose, mounted on formvar-carbon-coated gold EM grids, and rinsed with PBS.

Immunolabeling of antigens in the ultra-thin sections were performed using the protein A-gold technique (Slot and Geuze, 1983). Briefly, the sections were incubated in 10 mM NH₄Cl in PBS for 10 min and blocked in 0.1% fetal bovine serum for 10 min. The sections were then incubated for 30 min with monoclonal anti-furin (provided by Prof. A. White) and a rabbit anti-furin antibody (provided by Dr. W. Garten, Marburg, Germany) diluted in PBS containing 5% fetal calf serum and 0.1% BSA followed by washing with PBS/0.1% BSA (6 × 3 min). Rabbit antibodies were detected with protein A conjugated to 10 nm colloidal gold (Department of Cell Biology, University of Utrecht) in PBS/0.1% BSA. Sorting of Furin/PC2 Chimeras—PK15 cells were pre-infected with a recombinant vaccinia virus expressing T7 RNA polymerase (Creemers et al., 1993). A cDNA encoding the precursor of the von Willebrand factor (pro-vWF) together with cDNAs encoding furin, PC2, F-S, F-S-P, or F-N-P (all cloned in pcDNA3) were then transiently co-transfected (2 × 1 μg of DNA + 6 μl of lipofectAMINE (Life Technologies, Inc.,)). Analyses of newly synthesized vWF polypeptides was performed by SDSPAGE as reported previously (Creemers et al., 1993). The experiments were performed twice.
mutant has a wild-type furin middle domain except for Tyr$^{571}$ → Val substitution as a result of the SpeI splicing of the PC2 carboxyl-terminal domain. Otherwise, the junctional sequences were of the corresponding wild-type.

Three additional constructs were made: a PC2 mutant truncated after the middle domain (Thr$^{594}$), a chimera composed of the pro- and catalytic domains of PC2 and the middle, cysteine-rich, transmembrane and cytoplasmic domains of furin, and an equivalent chimera in which the middle domain was derived from PC2. These constructs were tested in parallel with those shown in Fig. 1. Although well expressed, neither propeptide cleavage nor substrate processing activity could be detected. Furthermore, the carbohydrate modifications on these chimeras remained endo H sensitive; no secretion was observed, and immunofluorescence microscopy indicated that the proteins did not exit the ER. These constructs were therefore uninformative with regard to sorting and were not considered further in this report.

The mouse pituitary tumor cell line AtT-20 was stably transfected with the constructs F-S, F-S-P, and F-N-P using the mammalian expression vector pcDNA3 to generate the cell lines AtT-20/F-S, AtT-20/F-S-P, and AtT-20/F-N-P. Act-20/ furin and AtT-20/PC2 were generated in the same experiments using wild-type furin and PC2 without the restriction site modifications. The AtT-20/PC2 cells remained clonal for an extended period of time. In contrast, the other cell lines could only be passaged for a few generations before expression of the transfected protein ceased. We speculate that overexpression of furin catalytic activity adversely affects AtT-20 cells resulting in negative selection of expressing cells despite maintenance of G418 resistance. All experiments with these cell lines were therefore performed with cells of a low passage number.

**RESULTS**

**Stable Transfection of AtT-20 Cells**—A schematic representation of the recombinant proteins used in this study is given in Fig. 1. To obtain these constructs, two unique restriction sites were used: a NarI site, which is present in native FUR cDNA, and a SpeI site, which was introduced in both cDNAs. Only in the case of the SpeI site in PC2 cDNA did the mutations lead to an amino acid substitution (Met$^{596}$ → Val) in the original sequence. This was conservative both in the physicochemical sense and in that many members of the subtilisin family including Lymnaea (Smit et al., 1992) and Aplysia (Quinet et al., 1993) PC2s have a Val residue in this position. The NarI sites are located in the region encoding the highly conserved carboxyl terminus of the subtilisin-like catalytic domains of furin family members. The carboxyl termini are defined as the residues equivalent to the carboxyl terminus of prokaryotic subtilisin-like enzyme thermolysin (Val$^{145}$ for furin and Val$^{153}$ for PC2; Siezen et al., 1994). The SpeI sites were introduced in the region encoding the partially conserved carboxyl termini of the middle domains (Thr-Leu-Val-Leu-Tyr-Gly-Thr$^{571}$ for furin, Thr-Leu-Met-Leu-His-Gly-Thr$^{594}$ for PC2). The truncated furin mutant F-S has an amber stop codon after Thr$^{573}$. The F-S-P

---

**Fig. 1. Schematic representation of the construction of chimeric and truncated proteins.** Amino acid substitutions are underlined. The asterisk indicates the stop codon. The various pro domains are indicated. CRR, cysteine-rich region; TM, transmembrane domain. The relative positions of N-linked glycosylation sites are indicated with +. Proteins are drawn roughly in scale. Scale bar indicates 100 aa.
Sorting of Furin/PC2 Chimeras

The truncated form of furin expressed in AtT-20/F-S cells (Fig. 2) was synthesized as a protein of 66 kDa and rapidly processed into a 57-kDa form. Both forms were endo H-sensitive. During chase experiments, partial conversion of the 57-kDa form into a 62-kDa endo H-resistant protein was observed. Only the 62-kDa form was secreted.

Similar maturation patterns were observed for the chimeric proteins expressed in AtT-20/F-S-P and AtT-20/F-N-P cells (Fig. 2). Both proteins were synthesized as precursors of 72 kDa, which were rapidly converted into 66- and 64-kDa forms, respectively. F-S-P was then converted into a higher molecular mass form of 72 kDa, while F-N-P did not increase in size during chase incubations. The secreted form of F-S-P was endo H-resistant, while the secreted form of F-N-P had both endo H-sensitive and -resistant oligosaccharide chains, the endo H-sensitive chain presumably located in the middle domain derived from PC2.

For AtT-20/PC2 and AtT-20/F-N-P cells, the major release of radiolabeled material occurred in the period 30–60 min post-labeling and declined thereafter despite the maintained presence of the mature protein in the cell. This is consistent with an initial wave of constitutive release of the mature forms of the protein accompanied by intracellular storage in a secretory granule compartment. In the case of F-S and F-S-P, release again occurred after a lag of around 30 min, but there was little evidence of intracellular storage as indicated by the progressive depletion of intracellular forms over the 60–360-min chase incubation. In AtT-20/furin cells, release was delayed for 120 min but thereafter increased progressively while the intracellular form declined in a reciprocal manner. Release in this instance was attributed to failure of the retention/retrieval mechanism which localizes it to the TGN as a consequence of the truncation of the carboxyl-terminal domain of the protein (Bosshart et al., 1994; Molloy et al., 1994; Schäfer et al., 1995).

Substrate Processing Activity of the Recombinant Enzymes—Activity studies were performed in transiently transfected PK(15) cells using the precursor of pro-vWF as substrate (Fig. 3). Little endogenous processing of pro-vWF was evident (lane 1); however, co-transfection with furin, F-S, F-S-P, or F-N-P (lanes 2–5) resulted in complete conversion to the mature form. PC2 is not active in this assay (De Strooper et al., 1995) but could be demonstrated using partially purified PC2 from AtT-20/PC2 cells and proinsulin as substrate (data not shown).²

Intracellular Localization of Recombinant Proteins—Indirect immunofluorescence microscopy showed that wild-type furin expressed in AtT-20/furin cells (Fig. 4A) co-localized with endogenous TGN38 (Fig. 4B), a marker protein for the TGN (Luzzio et al., 1990). Recombinant PC2, on the other hand (Fig. 4C), was present in the neurite extensions, where it co-localized with POMC immunoreactivity (Fig. 4D). Diffuse cytoplasmic staining was also evident, possibly reflecting newly synthesized protein localized in the ER as a result of the relatively slow exit rate from that compartment (Guest et al., 1992; Benjannet et al., 1993; Zhou and Mains, 1994). The punctate staining throughout the cytoplasm, which was observed by anti-POMC antibodies, was largely masked by this ER-like staining.

Weak cytoplasmic staining for F-S was observed in AtT-20/F-S cells (Fig. 4E). It resembled the staining obtained with an antibody directed against the Lys-Asp-Glu-Leu (Napier et al., 1988) sequence, which is found on luminal ER-resident proteins (Fig. 4F), and suggested localization to that compartment. This is consistent with rapid secretion of this truncated furin mutant lacking a transmembrane domain and the observation that the major cellular form of the protein was the endo H-sensitive precursor.

The majority of staining of the chimeric protein F-S-P was diffusely distributed in the cytoplasm with a second component co-localizing with POMC immunoreactivity in punctate structures, most notably in neurite-like extensions of the cell (Fig. 4, G and H). Such granular staining was not particularly intense, which was consistent with the relatively poor retention of the protein in the cell observed in pulse-chase labeling experiments.

The F-N-P chimera expressed in AtT-20/F-N-P cells (Fig. 4I) was similarly found in POMC-immunoreactive vesicles (Fig. 4J).

² E. M. Bailyes, personal communication.
both in the neurites and the cytoplasm of the cell. The granular staining was more intense than in the case of F-S-P consistent with the greater retention of the protein as observed in pulse-chase labeling studies.

**Regulated Secretion of Recombinant Proteins**—The secretory response of transfected AtT-20 cells to the secretagogues forskolin and IBMX are shown in Table I. The release of PC2 was more than doubled by the secretagogues (2.5-fold) in contrast to F-S, which showed no response (1.04-fold), consistent with the targeting of the former protein to the granule compartment and the constitutive release of the latter. In the same assay, secretion of mutant F-S-P was enhanced 1.5-fold, whereas a more pronounced effect of forskolin was observed in the case of F-N-P (1.9-fold), although the response in this case did not reach the magnitude of that of PC2. This apparent ranking of these responses paralleled the observations made by immunofluorescence microscopy and pulse-chase labeling experiments in suggesting that PC2 and the F-N-P chimera were stored efficiently, whereas F-S-P, although bearing a targeting signal for the regulated pathway, was less efficiently sorted into the granule compartment. This was also evident from the rate of basal release under the various transfection conditions; thus, F-S showed the highest relative rate of release, wild-type PC2 showed the lowest, and F-S-P and F-N-P were ranked in the anticipated order between these extremes.

**Immunoelectron Microscopic Analysis of Recombinant Proteins**—Immunoelectron microscopic studies were performed to investigate the nature of the compartment in which F-S-P and F-N-P were retained in the cell (Fig. 5). Immunolabeling of thin frozen sections with anti-furin antibodies (Fig. 5A) showed the expected localization of the protein in the TGN. Occasional gold particles, however, were also found on the periphery of secretory granules for this protein (Fig. 5A, inset). Immunolabeling of AtT-20/F-S-P and AtT-20/F-N-P cells demonstrated the presence of these chimeras in organelles with a typical dense-core secretory granules morphology (Fig. 5, B and C, respectively) that greatly exceeded that observed above in AtT-20/furin cells prepared at the same time using the same antibody preparation. Several granules in both cell lines, however, contained no gold particles, which raised the issue of whether these proteins were targeted to a subset of granules or to a related organelle.

![Fig. 4. Intracellular localization of the recombinant proteins in stable transfected AtT-20 cells.](image-url)

**FIG. 4.** Intracellular localization of the recombinant proteins in stable transfected AtT-20 cells. Cells were fixed and used for immunofluorescence analysis as described under "Materials and Methods." Cells were double-labeled with antibodies directed against the recombinant protein and a marker protein for the ER (Lys-Asp-Glu-Leu-containing proteins), TGN (TGN38), or secretory granules (POMC) as indicated. A and B, AtT-20/furin; C and D, AtT-20/PC2; E and F, AtT-20/F-S; G and H, AtT-20/F-S-P; I and J, AtT-20/F-N-P. Scale bar represents 10 μm.

| Construct | Control | Stimulated |
|-----------|---------|------------|
| PC2       | 10.7 ± 3.8 | 26.4 ± 5.3 |
| F-S       | 34.9 ± 3.4 | 36.4 ± 4.5 |
| F-S-P     | 23.5 ± 3.1 | 36.3 ± 4.8 |
| F-N-P     | 19.8 ± 7.9 | 33.1 ± 6.8 |

**TABLE I**

Secretion of recombinant proteins in transiently transfected AtT-20 cells

After transfection, cells were cultured overnight in serum-free medium with 10 μM Rp-cAMP. The next day the cells were incubated 3 h with the secretagogues forskolin (10μM) and IBMX (0.1 mM) or with control medium. Cell lysates and medium were analyzed as described under “Materials and Methods.” The percentage is the relative amount of protein secreted (amount secreted/total amount × 100%). Results are the mean ± S.E. of the results of five independent transfection experiments.

![Fig. 5. Immunogold labeling of thin frozen sections of AtT-20/furin, AtT-20/F-S-P, and AtT-20/F-N-P.](image-url)

**FIG. 5.** Immunogold labeling of thin frozen sections of AtT-20/furin, AtT-20/F-S-P, and AtT-20/F-N-P. Cells were fixed and used for immunoelectron microscopy as described under “Materials and Methods.” Rabbit anti-furin antibody was detected with protein A conjugated to 10 nm (A-C) or 15 nm (D) of colloidal gold. Mouse anti-POMC antibody was detected with goat anti-mouse IgG conjugated to 10 nm of gold (D). A, AtT-20/furin; B, AtT-20/F-S-P; C and D, AtT-20/F-N-P. Golgi stacks are indicated with "G." Scale bar represents 200 nm.
of similar morphology. Double labeling experiments in AtT-20 F-N-P cells using anti-furin (15 nm of gold) and anti-POMC (10 nm of gold) antibodies (Fig. 5D) showed that most granules were decorated with multiple 10 nm of gold particles (POMC) and that F-N-P, when it was observed, was only associated with granules containing POMC immunoreactivity. The apparent co-packaging of POMC-related peptides and F-N-P was further evidenced by the appearance of both markers in favorable sections at the trans-face of the Golgi (Fig. 5D, inset). The observation that several granules were only labeled with 10 nm of gold particles is attributed to a concentration effect rather than the result of differential targeting between POMC and this chimera.

**DISCUSSION**

Previous transfection studies using a range of different cell lines and vector systems have indicated that newly synthesized pro-furin is rapidly and completely cleaved into furin in the ER by an autocatalytic intramolecular process (Creemers et al., 1993, 1995). N-Linked oligosaccharide chains are subsequently modified, and the protein takes up residence in the TGN where it is maintained by a combined retention/retrieval mechanism (Bosshart et al., 1994; Molloy et al., 1994; Schäfer et al., 1995). In NRK cells, it may eventually be truncated in its carboxy-terminal region and released into the medium (Vey et al., 1994). In RBL cells, it is reported to be degraded in a population of hybrid organelles with lysosomal characteristics (Bosshart et al., 1994). In this study conducted in stable transfected AtT-20 cells, immunogold electron microscopy revealed that the steady state localization of furin was principally the TGN. The presence of gold particles on the periphery of a few secretory granules in AtT-20/furin cells, however, raised the question of whether or not the molecule is totally excluded from the granule membrane during granule biogenesis.

The maturation of PC2 in the stable transfected AtT-20 cells was consistent with the reported proteolytic cleavage of pro-PC2 at both tetrabasic sites Lys-Arg-Arg-Arg81 or Arg-Lys-Lys-Arg109 to produce the mature secreted forms (Bailyes et al., 1995). The intracellular localization of propeptide cleavage has not been determined unequivocally, although the present consensus favors a late compartment, probably the TGN or immature granule. In our stable transfected AtT-20 cells, the constitutive release of a substantial amount of mature PC2 but little pro-PC2 argues that processing occurs before sorting. The processed form of PC2 was shown to contain both endo H-sensitive and endo H-resistant carbohydrate side chains, whereas pro-PC2 was completely endo H-sensitive, as reported previously (Guest et al., 1992; Benjannet et al., 1993; Mains et al., 1995). This superficially suggests that pro-PC2 does not reach the trans-Golgi. Against such a suggestion are previous reports that a component of pro-PC2 is tyrosine sulfated (Benjannet et al., 1992, 1993; Mains et al., 1995). The truncation of furin after the middle domain (Thr273) resulted in the production of an active, secreted enzyme that underwent post-translational modification with similar kinetics to the wild-type molecule. The smallest active form of furin described previously was truncated after Glu576 (Hatsuzawa et al., 1992), while the largest inactive mutant was truncated after Glu566 (Creemers et al., 1993). Truncation of yeast kexin at the position equivalent to furin Thr273 also generates an active enzyme (Glschankof and Fuller, 1994), whereas the removal of one additional carboxy-terminal amino acid blocks activation and results in ER retention. The implication that the carboxy-terminal amino acid residues of the middle domain are critically important in folding, autoactivation, and/or intracellular transport has possible bearing on the failure to produce an active truncated form of PC2 (truncated at the position equivalent to F-S), which bores a single conservative amino acid substitution 5 aa from its carboxyl terminus (Met566 → Val). It may also be that additional residues within the carboxy-terminal domain of PC2 are required for autonomous function of the middle domain of PC2. Further mutagenesis studies could resolve this issue.

The addition of the last 50 aa of PC2 to the truncated form of furin did not markedly affect the rate of propeptide cleavage, intracellular transport, or the catalytic activity of the molecule. The other chimera F-N-P also matured normally, showing that the middle domains of furin and PC2 can be exchanged and are functionally equivalent with respect to effects on folding of the proprotein and its transport to the TGN. It is concluded from these results that the strategy of swapping mammalian subtilisin domains to explore the location and function of targeting motifs is informative. Since PC2 is more distantly related to furin than any other member of the mammalian subtilisin-like gene family, it follows that this strategy could be extended to middle domain exchanges with other members of the family. Similarly successful domain-swapping experiments were performed by Zhou et al. (1995) in a study of the role of the propeptides of furin, PC1, and PC2 in routing and endoproteolytic activity. Other studies of this kind (Rehemtulla et al., 1992), however, have generated inactive constructs.

As shown previously (Bosshart et al., 1994; Molloy et al., 1994; Schäfer et al., 1995), the deletion of the carboxyl-terminal domains in F-S resulted in its constitutive secretion. Intracellular furin immunoreactivity was only associated with the ER, presumably reflecting the transient retention of newly synthesized molecules prior to folding. Within the context of the behavior of the chimeric molecules, these observations suggest that the catalytic and middle domains of the furin do not possess sub-dominant motifs that could target the molecule to granules or other intracellular organelles. Studies of the localization of chimeric proteins, on the other hand, indicated a critical role for the last 50 aa of PC2 in the sorting to the secretory granule compartment. The carboxyl terminus of PC2 is predicted to contain an amphipathic α-helical segment (Smeekens et al., 1991), and similar structures have been im-
plicated in membrane association and sorting of carboxypeptidase H (CPH) (Mitra et al., 1994). Addition of the CPH tail to albumin results in rerouting of albumin from the constitutive to the regulated pathway in AtT-20 cells. As in our experiments, sorting of the albumin/CPH chimera was less efficient than sorting of CPH, suggesting that although sorting may largely depend on a specific domain, additional components of the protein structure may contribute. Carboxyl-terminal truncation of PC1 (which also has a putative amphiphatic α-helical segment (Smeekens et al., 1991)) results in the synthesis of a functional enzyme, the majority of which is not sorted into granules (Zhou et al., 1995a, 1995b). Nevertheless, some activity of the truncated PC1 within the regulated pathway was detectable, suggesting that additional sorting domains may exist in PC1.

On the basis of an alignment of amphiphatic α-helices of 15 other proteins that are sorted to the granules, Kizier and Tropsha (1991) postulated a degenerate sorting motif consisting of several leucines and a serine or a threonine. None of the carboxyl termini of PC2, PC1, or CPH conform to this motif, and several leucines and a serine or a threonine. None of the carboxyl termini of PC2, PC1, or CPH conform to this motif, and several leucines and a serine or a threonine. Hence, it seems unlikely that this motif plays a significant role in the sorting of these proteins. Nevertheless, some activity of the truncated PC1 within the regulated pathway was detectable, suggesting that additional sorting domains may exist in PC1.

In contrast to the present findings, other studies with regulated secretory proteins, particularly somatostatin and POMC, have implicated the pro-region in sorting into the regulated pathway (Stoller and Shields, 1989; Cool et al., 1995). PC2 expressed in micro-injected Xenopus oocytes (Shennan et al., 1994) shows Ca\(^2\)- and pH-dependent aggregation and membrane association, which is dependent on the propeptide but independent of its carboxyl terminus. These results have been interpreted as evidence for a sorting signal in the pro-region; however, this appears unlikely in view of the finding that the pro-sequence of PC2 is cleaved prior to sorting and the fact that these specific sequences were absent in our chimeraus. Such aggregation and membrane association of pro-PC2 may impact more on its behavior in the ER than in the TGN, particularly in view of the report by Mains et al. (1995) that modification of the propeptide of PC2 influences its rate of exit from the ER.

Our studies point to an important contribution of the middle domain of PC2 in promoting the efficiency of the sorting process. It is conceivable that this domain possesses an independent sorting signal or that the carboxyl-terminal domain sorting motif incorporates components of the structure of the middle domain or even overlaps with it. The middle domain might also provide a scaffold for the correct conformational expression of a sorting domain within the carboxyl-terminal region of the molecule. Examination of the sequence alignment of the middle domains of all the currently documented forms of furin, PC2, and PC1 molecules did not reveal significant differences between furin and the PCs or unique common sequences between PC1 and PC2, which could be responsible for enhancement of the efficiency of sorting of F-N-P as compared to F-S-P.

How the sorting elements in the carboxyl terminus and middle domain of PC2 function in a cellular context is not clear from the present studies. Of the current models of receptor-mediated (Chung et al., 1989), aggregation-based (Kelly, 1985; Chanat and Huttner, 1991), and passive sorting (Kulawat and Arvan, 1992), only passive sorting is not favored since it would predict that the F-S construct would behave similarly to F-S-P and F-N-P. If sorting of PC2 were to occur by self-association alone, it is difficult to envisage how the enzyme, which is only a small fraction of the granule cargo, would be uniformly distributed within and between secretory granules. Although a purely receptor-based mechanism presents a conceptual difficulty for the sorting of major secretory products, particularly with regard to the receptor-ligand stoichiometry, it could easily operate with minor products such as PC2. The "receptor" moreover need not be membrane associated and could in fact be the pro-form of the major secretory product itself. The prohormone POMC in AtT-20 cells is a substrate for PC2 and furin and is probably present at a concentration several orders of magnitude higher than the Km of the enzyme and the concentration of the enzyme. Efficiency of sorting might be expected to be related to the kinetic properties of the enzyme and in particular its substrate specificity. However, in the present instance, this would not account for why F-S-P and F-N-P but not F-S were sorted to the regulated pathway since these were correctly folded and active. Therefore, it is concluded that the carboxyl terminus of PC2 provides some specific signal, be it a ligand for a sequence-specific receptor or an element promoting self-association, which directs the molecule toward the regulated pathway of secretion. The physicochemical characteristics of the middle domain of PC2 might further enhance such processes by providing a structural context or might possess further elements that independently promote sorting.

Acknowledgments—We thank Dr. E. M. Bailyes and Dr. A. J. M. Roebroek for helpful discussion and critical reading of the manuscript.

REFERENCES

Bailyes, E. M., Shennan, K. J. J., Usac, E. F., Arden, S. D., Guest, P. C., Docherty, K., and Hutton, J. C. (1995) Biochem. J. 309, 587–594
Benten, N., Rondeau, N., Paquet, L., Boudreault, A., Lazure, C., Chrétien, M., and Seidah, N. G. (1993) Biochem. J. 294, 735–743
Booshart, H., Humphrey, J., Deignan, E., Davidson, J., Drabza, J., Yuan, L., Oorschot, V., Peters, P. J., and Bonifacino, J. S. (1994) J. Biol. Cell. 126, 1157–1172
Chanat, E., and Huttner, W. B. (1991) J. Cell Biol. 115, 1505–1519
Chung, K. N., Walter, P., Aponte, G. W., and Moore, H. P. (1989) Science 243, 198–207
Cool, D. R., Fenger, M., Snell, C. R., and Loh, Y. P. (1995) J. Biol. Chem. 270, 5723–5729
Creemers, J. W. M., Siezen, R. J., Roebroek, A. J. M., Ayoubi, T. A. Y., Huybreelbroek, D., and Van de Ven, W. J. M. (1993) J. Biol. Chem. 268, 21836–21834
Creemers, J. W. M., Vey, M., Schafer, W., Ayoubi, T. A. Y., Roebroek, A. J. M., Kleek, H. D., Garten, W., and Van de Ven, W. J. M. (1995) J. Biol. Chem. 270, 26967–26972
De Strooper, B., Creemers, J. W. M., Moechars, D., Huybreelbroek, D., Van de Ven, W. J. M., Van Leuvren, F., and Van den Bergh, H. (1995) Biochim. Biophys. Acta 1258, 155–158
Gluschankof, P., and Fuller, R. S. (1994) EMBO J. 13, 2280–2288
Griffiths, G. (1993) Fine Structure Immunocytochemistry, Springer-Verlag, Berlin
Guest, P. C., Arden, S. D., Bennett, D. L., Clark, A., Rutherford, N. G., and Hutton, J. C. (1992) J. Biol. Chem. 267, 22401–22406
Halan, P. A., and Irmlinger, J. C. (1994) Biochem. J. 299, 1–18
Hashimoto, S., Fumagalli, G., Zanini, A., and Meldolesi, J. (1987) J. Cell Biol. 105, 1579–1586
Hatsuzawa, K., Murakami, K., and Nakayama, K. (1992) J. Biochem. (Tokyo) 111, 296–301
Hutton, J. C. (1990) Curr. Opin. Cell Biol. 2, 1131–1142
Kelly, R. B. (1985) Science 230, 25–32
Kizier, J. S., and Tropsha, A. (1991) Biochem. Biophys. Res. Commun. 174, 596–592
Kulawat, R., and Arvan, P. (1994) J. Biol. Chem. 269, 118, 512–519
Luzio, J. P., Brake, B., Banting, G., Howell, K. E., Braggilotta, H. P., and Stanley, K. K. (1999) Biochem. J. 270, 97–102
Mains, R. E., Milgram, S. L., Keutmann, H. T., and Eipper, B. A. (1995) Mol. Endocrinol. 9, 1–13
Malide, D., Seidah, N. G., Chrétien, M., and Bendayan, M. (1995) J. Histochem. Cytochem. 43, 11–19
Mitra, A. Seng, L., and Fricker, L. D.(1994) J. Biol. Chem. 269, 19876–19881
Molloy, S. S., Thomas, L., Van Slyke, J. K., Stenberg, P. E., and Thomas, G. (1994) EMBO J. 13, 18–33
Moore, H. P., and Kelly, R. B. (1986) Nature 321, 443–446
Mror, E. A., and Lechene, C. (1986) Science 232, 871–873
Napier, R. M., Venis, M. A., Bolten, M. A., Richardson, L. J., and Butcher, G. W. (1988) Planta 176, 519–526
Ouellet, T., Mammarbachi, A., Cloutier, T., Seidah, N. G., and Castellucci, V. F. (1993) FEBS Lett. 330, 343–346
Parkinson, D. (1990) J. Biol. Chem. 265, 17101–17105
Rhemtulla, A., Donner, A. J., and Kaufman, R. J. (1992) Proc. Natl. Acad. Sci.
Sorting of Furin/PC2 Chimeras

Steiner, D. F., Smeal, S. P., Ohagi, S., and Chan, S. J. (1992) J. Biol. Chem. 267, 22435–22438
Stoller T. J., and Shields, D. (1989) J. Cell Biol. 108, 1647–1655
Tokuyasu, K. T. (1978) J. Ultrastruct. Res. 63, 287–307
Touze, S. A., Hollinshead, M., and Dittie, A. S. (1994) Biochimie (Paris) 76, 271–276
Van den Ouweland, A. M. W., Van Duijnhoven, J. L. P., Keizer, G. D., Dorsers, L. C. J., and Van den Ve, W. J. M. (1990) Nucleic Acids Res. 18, 664
Van de Ven, W. J. M., Voorberg, J., Pannenhoek, H., Van den Ouweland, A. W. M., and Siezen, R. J. (1990) Mol. Biol. Rep. 14, 265–275
Van de Ven, W. J. M., Van Duijnhoven, J. L. P., and Roebroek, A. J. M. (1993) Crit. Rev. Oncogen. 4, 115–136
Van Duijnhoven, J. L. P., Creemers, J. W. M., Kranenborg, M. G. C., Timmer, E. D. J., Groeneveld, A., Van den Ouweland, A. M. W., Roebroek, A. J. M., and Van de Ven, W. J. M. (1992) Hybridoma 11, 71–86
Vey, M., Schäfer, W., Berghöfer, S., Klenk, H.-D., and Garten, W. (1994) J. Cell Biol. 127, 1829–1842
Zhou, A., Paquet, L., and Mains, R. E. (1995a) J. Biol. Chem. 270, 21509–21516
Zhou, Y., Rovere, C., KitaIgi, P., and Lindberg, I. (1995b) J. Biol. Chem. 270, 24792–24796
Zhu, X., and Lindberg, I. (1995) J. Cell Biol. 129, 1641–1650

Steiner, D. F., Smeal, S. P., Ohagi, S., and Chan, S. J. (1992) J. Biol. Chem. 267, 22435–22438
Stoller T. J., and Shields, D. (1989) J. Cell Biol. 108, 1647–1655
Tokuyasu, K. T. (1978) J. Ultrastruct. Res. 63, 287–307
Touze, S. A., Hollinshead, M., and Dittie, A. S. (1994) Biochimie (Paris) 76, 271–276
Van den Ouweland, A. M. W., Van Duijnhoven, J. L. P., Keizer, G. D., Dorsers, L. C. J., and Van den Ve, W. J. M. (1990) Nucleic Acids Res. 18, 664
Van de Ven, W. J. M., Voorberg, J., Pannenhoek, H., Van den Ouweland, A. W. M., and Siezen, R. J. (1990) Mol. Biol. Rep. 14, 265–275
Van de Ven, W. J. M., Van Duijnhoven, J. L. P., and Roebroek, A. J. M. (1993) Crit. Rev. Oncogen. 4, 115–136
Van Duijnhoven, J. L. P., Creemers, J. W. M., Kranenborg, M. G. C., Timmer, E. D. J., Groeneveld, A., Van den Ouweland, A. M. W., Roebroek, A. J. M., and Van de Ven, W. J. M. (1992) Hybridoma 11, 71–86
Vey, M., Schäfer, W., Berghöfer, S., Klenk, H.-D., and Garten, W. (1994) J. Cell Biol. 127, 1829–1842
Zhou, A., Paquet, L., and Mains, R. E. (1995a) J. Biol. Chem. 270, 21509–21516
Zhou, Y., Rovere, C., KitaIgi, P., and Lindberg, I. (1995b) J. Biol. Chem. 270, 24792–24796
Zhu, X., and Lindberg, I. (1995) J. Cell Biol. 129, 1641–1650