Biochemical and enzymatic characterization of the novel proprotein convertase rat PC7 (rPC7) was carried out using vaccinia virus recombinants overexpressed in mammalian BSC40 cells. Pro-PC7 is synthesized as a glycosylated zymogen (101 kDa) and processed into mature rPC7 (89 kDa) in the endoplasmic reticulum. No endogenously produced soluble forms of this membrane-anchored protein were detected. A deletion mutant (65 kDa), truncated well beyond the expected C-terminal boundary of the P-domain, produced soluble rPC7 in the culture medium. Enzymatic activity assays of rPC7 using fluorogenic peptideyl substrates indicated that the pH optimum, Ca²⁺ dependence, and cleavage specificity of this enzyme are largely similar to those of furin. However, with some substrates, cleavage specificity more closely resembled that of yeast kexin, suggesting differential processing of proprotein substrates by this novel convertase. We examined the rPC7- and human furin-mediated cleavage of synthetic peptides containing the processing sites of three proteins known to colocalize in situ with rPC7. Whereas both enzymes correctly processed the pro-parathyroid hormone tricapeptide and the pro-PC4 heptadecapeptide, neither enzyme cleaved a pro-epidermal growth factor hexadecapeptide. Thus, this study establishes that rPC7 is an enzymatically functional subtilisin/kexin-like serine proteinase with a cleavage specificity resembling that of furin.

In addition, we have demonstrated that rPC7 can correctly process peptide precursors that contain the processing sites of at least two potential physiological substrates.

Mammalian prohormone convertases comprise a family of serine proteinases whose function is the cleavage of peptide precursor molecules at distinct single or pairs of basic residues (1, 2). These enzymes are related to bacterial subtilisins and to the yeast prohormone processing protease kexin. There are presently seven known members of this family that have been grouped under the generic name proprotein convertases (PCs).

These include furin (also known as PACE), PC1 (also known as PC3), PC2, PACE4, PC4, PC5 (also known as PC6) (for reviews, see Refs. 1–4), and, most recently, PC7 (also known as LPC (6), PC8 (7), or SPc7 (8)). Tissue distribution analyses of these enzymes indicate that PC1 and PC2 are expressed mainly in neural and endocrine tissues, PC4 exclusively in reproductive germ cells, and PC5 and PACE4 to varying degrees in many tissue types (1–3). Similar to furin (3, 4), PC7 has a nearly ubiquitous tissue distribution as assessed by mRNA expression (5, 7, 8), suggesting that it could, like furin (4), be involved in the processing of precursors within the constitutive secretory pathway. Interestingly, in lymphoid-associated tissues such as thymus, spleen, and lymph nodes, the expression of PC7 appears to be especially high (5).

The sites where PCs cleave peptide precursors of various hormones, growth factors, and viral envelope glycoproteins have generally been defined as (Arg/Lys)-(X)ₙ-(Arg/Lys) where n = 0, 2, 4, or 6 residues (9–11). Due to this wide-ranging specificity and the overlapping expression of various convertases in different tissues, it is often difficult to assign cleavage of a given precursor to a particular enzyme. Although it is clear that PC1, PC2 (12–14), and PC5-A (15) are the enzymes most likely to be active in secretory vesicles (9), processing within the compartments of the early secretory pathway may be best performed by furin, PACE4, PC5-B (16, 17), and, most likely, PC7.

This paper characterizes the in vitro properties of full-length and soluble rat PC7 (rPC7) overexpressed in mammalian cells infected with the corresponding vaccinia virus (VV) recombinants. A polyclonal rPC7 antiserum has been produced and used to identify the biosynthetic forms, likely cellular location, and site of zymogen cleavage of rPC7 in these cells. In addition, processing of several synthetic fluorogenic and peptideyl substrates is examined to elucidate the cleavage specificity of this newest member of the PC family.

**Experimental Procedures**

Vaccinia Virus Constructs—The full-length cDNA of rPC7 (5) that had been inserted into the pBluescript vector (Stratagene) was digested with (5′) HindIII and (3′) BsoI to remove a pair of extra 5′ ATG codons (5). Following the addition of a linker to close the sequence, the insert (5′) HindIII/(3′) XhoI was ligated into the HindIII/NheI sites of the VV transfer vector PMJ602 (18). This produced the rPC7 full-length recombinant virus (VV:rPC7). The native initiation sequence of this construct, CTGATGCG, was subsequently modified by polymerase chain reaction to GTGATGG to generate a favorable Kozak consensus sequence for the initiation of protein translation (19).

Three soluble forms of rPC7 were constructed from this full-length VV:rPC7 clone by digestion with restriction enzymes followed by addition of the appropriate linkers. Thus, digestion with BsmBI produced a form called BTMD (before the transmembrane domain) rPC7, which

ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse phase-high performance liquid chromatography; MBS, 4-morpholineethanesulfonic acid; pfu, plaque-forming units.
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ends at Gly-Tyr-Ser-Glu. Similarly, digestion with HglA generated two shorter forms of rPC7, called BTMD-S-rPC7, ending at Tyr-Gly-Ser and at Val-Asp-Ile-Glu. Vaccinia virus recombinants of the three constructs were then isolated as described previously (12, 14). Human BCRD-prepared membranes containing 10% Triton X-100 (v/v) in the case of membrane-bound enzyme preparations, as well as the pulse periods, the concentrations of tunicamycin and charged on an Applied Biosystem gas-phase sequenator (model 470A) as described previously (12, 25).

Enzymatic Activity Determinations—Enzymatic activity was assayed using the fluorogenic synthetic peptide substrate pERTKR-MCA (Peptides International) (26) as follows. The enzyme preparation (usually 5 μl) was added to the assay reaction mixture consisting of (final concentration) 50 mM Tris-Cl, pH 7.4, 5 mM CaCl2, 1 mM EDTA, 0.5% Triton X-100 (v/v) in the case of membrane-bound enzyme preparations, and 50 μM 2-mercaptoethanol in the case of BCRD-hfurin. The reaction (in a total volume of 100 μl) was initiated at room temperature in a microtiter plate by the addition of pERTKR-MCA at a final concentration of 0.1 mM. Fluorescence was monitored at 0, 30, 60, and 90 min using a microfluorometer (model LS 50 B) set to an excitation wavelength of 370 nm and an emission wavelength of 460 nm. In all cases, the rate of substrate hydrolysis was constant for at least 60 min.

pH, Ca2+, and Inhibitor Profiles—For pH sensitivity experiments, enzymatic activity was evaluated as above using a ternary buffer system containing 17.3 mM sodium acetate, 17.3 mM MES, and 17.3 mM Tris acetate adjusted to the appropriate pH with acetic acid or NaOH. To determine the Ca2+ activation requirement of PC7, increasing concentrations of EDTA were added to the assay mixture described above for enzymatic activity measurements but lacking CaCl2. Based on these results, the assay mixture was then supplemented with 1 mM EDTA and varying concentrations of CaCl2. For the inhibitor profiles, selected compounds (for the recombinant human and yeast enzymes) were added to the assay mixture described above before the addition of pERTKR-MCA. In the case of EDTA and EGTA, the indicated concentrations of the chelator were added to the assay mixture without CaCl2.

Fluorogenic Substrates and Km Determinations—Fluorogenic assays were carried out substituting various MCA substrates for pERTKR-MCA. For Km determinations, increasing amounts of pERTKR-MCA were added to the reaction mixture. The data, plotted as hydrolysis activity versus pERTKR-MCA, were subject to nonlinear regression analysis (KaleidaGraph) to determine the Km and Vmax values. For the representative assays depicted in the figures, the data were expressed as a percentage of the calculated Vmax and replotted.

Synthesis of Peptide Substrates—The pro-PC4 and pro-epidermal growth factor (pro-EGF) peptides were synthesized on an automated solid phase peptide synthesizer (Applied Biosystems, model 431A) using 2-(1H-benzo triazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate-based FastMoc chemistry (22, 23). The following side chain protecting groups were used: tert-butylcarbonyl for Lys; tert-butyloxycarbonyl for Tyr; Thr, Tyr, Asp, and Glu; and 2,2,5,7,8-pentamethylchroman 6-sulfonyle for Arg. At the end of the synthesis, the peptides were cleaved from the resin and fully deprotected by treating the resin with reagent K (22, 23) for 5 h, followed by lyophilization and repeated washings with ether. The peptides were purified by RP-HPLC (Beckman, model 5500) on a semi-preparative Vydac column 218TP510G8 (1.0 × 25 cm) (Chromatographic Specialty Corp., Canada). The buffer system consisted of an aqueous phase containing 0.1% trifluoroacetic acid (v/v) and an organic phase of acetonitrile containing 0.1% trifluoroacetic acid (v/v). The peptides were monitored at 215 nm and 254 nm. The peptides were identified using FAB mass spectrometry as well as by amino acid analysis (23). The synthetic pro-parathyroid hormone (pro-PTH) tridecapeptide was a gift from Dr. G. N. Hendy, Calcium Research Laboratory, Royal Victoria Hospital, McGill University, and has been described previously (27).

RP-HPLC Analysis of Synthetic Peptide Substrate Digests—The pro-PTH tridecapeptide (KVSKRRSVEIQ), the pro-PC4 heptadecapeptide (YELTRRRVKRSVLYVPPTD), and the pro-EGF hexadecapeptide (HLREDHSSVSNRS) were reacted at room temperature with enzymes in the reaction mixture as described above for enzymatic activity determinations. Time courses of substrate digestion by BCRD-hfurin, rPC7, and BTMD-rPC7 were first carried out to optimize the digestion conditions and to define the period during which the reaction proceeded at a constant rate (not shown). Thus, the reaction times chosen for the Km determinations were from 10 to 20 min. The digestion products were analyzed using RP-HPLC separation (Varian, model 9010) on a Beckman Ultrasphere C18 column (0.2 × 25 cm) as described above for peptide purification except that the buffer system also contained 0.01% triethylamine in both the aqueous and organic phases, the flow rate was 1 ml/min, the linear gradient of acetonitrile was 5–30% over 45 min, and monitoring was carried out at a wavelength of 210 nm. The collected peptides (two product peaks along with that of the undigested peptide, not shown) were identified and quantitated by amino acid analysis.
expressing either rPC7K or BTMD-rPC7K, the cell extracts, and overnight incubation of cells infected with VV recombinants obtained via overexpression in BSC40 cells. Thus, following identification and characterization of the various forms of rPC7 to obtain a polyclonal rPC7 antiserum, permitting the sequence indicated within the P-domain was used as an immunogen, called BCRD-hfurin. The 17-amino acid peptide sequence of the full-length rPC7 and three truncated versions lacking the putative transmembrane domain and cytosolic tail composition as described (27). The $K_m$ determinations were based on the amounts of the C-terminal cleavage product of each substrate peptide, and the resulting data were analyzed as described above for the pERTKR-MCA peptide.

**RESULTS**

Western Blot Analyses and In Vitro Enzymatic Activities of rPC7 and BTMD-rPC7—A schematic representation of the sequence of the full-length rPC7 and three truncated versions lacking the putative transmembrane domain and cytosolic tail (5), denoted BTMD-rPC7 and C-terminally shortened versions called BTMD-S-rPC7, is shown in Fig. 1. These molecular structures are compared with those of ykexin, the full-length hfurin, and its truncated version lacking the cysteine-rich domain, called BCRD-hfurin. The 17-amino acid peptide sequence indicated within the P-domain was used as an immunogen to obtain a polyclonal rPC7 antiserum, permitting the identification and characterization of the various forms of rPC7 obtained via overexpression in BSC40 cells. Thus, following overnight incubation of cells infected with VV recombinants expressing either rPC7K or BTMD-rPC7K, the cell extracts, and media were analyzed by Western blotting. As shown in Fig. 2, an rPC7-specific doublet (101 and 89 kDa, at a ratio of approximately 1:3 as determined by densitometric analysis of the ECL film) is visible only in the membrane fraction of cell lysates obtained from VV:rPC7-infected cells. (The construct VV:rPC7K produced 2–3 times more enzyme than VV containing the native initiator sequence, results not shown.) These two proteins were resistant to solubilization with 0.1 M Na$_2$CO$_3$, and even low (<5%) concentrations of Triton X-100 (not shown), indicating that rPC7 is an integral membrane protein. These findings were further supported by enzymatic activity assays of crude cellular fractions (Table I), which showed that 93% of the total rPC7-specific pERTKR-MCA hydrolytic activity was present in cell membranes. Analysis of BSC40 cells infected with VV:ykexin revealed that 21% of its activity is shed into the medium during a 7-h collection (subsequent to an overnight incubation), whereas 66% remains membrane-associated (Table I). Similarly, infections of BSC40 cells with VV:hfurin (full-length) resulted in the secretion of active, soluble furin (28, 29). These data thus confirm that rPC7, unlike hfurin and ykexin, remains as a membrane-associated protein which is not significantly shed into the medium.

In comparison, Western blot analysis of cells infected with VV:BTMD-rPC7K revealed an intracellular doublet in the membranes (77 and 65 kDa, at a ratio of about 5:1), as well as a 65-kDa form in the lysate supernatant and in the cell culture medium (Fig. 2). Densitometric quantitation and correction for the fractional amount deposited on the gel revealed that during a 7-h collection (subsequent to an overnight incubation), 99% of the total 65-kDa BTMD-rPC7 produced during this period was present in the cell culture medium. Similarly, upon measurement of the pERTKR-MCA cleavage activity of BTMD-rPC7 in *vitro*, we estimate that 89% of the total enzymatic activity is released into this 7-h medium (Table I). This value compares with the secretion of 100% of the activity of the soluble BCRD-hfurin (Table I). In contrast, neither form of BTMD-S-rPC7, comprising 578 or 585 amino acids (Fig. 1), was detectable by Western blotting or enzymatic activity measurements in the
BSC40 cells were pulse-labeled with [35S]Met for 10 or 30 min and then [3H]Leu-labeled. A 101-kDa form revealed a major sequence with a tryptic peptide containing an Ile2 sequence of the 89-kDa protein shows that its N terminus is likely to be at the sequence RAKR (5). We interpret these data to mean that the signal peptide cleavage site can occur either at the predicted (5) sequence (QVMG | LTEAG-GGLDTLGAGLSSLAVWY |) or at an alternative site four residues later (LTEA | GGLDTLGAGLSSLAVWY |). The Leu15 and Ile2 sequences of the 89-kDa protein show that its N terminus corresponds to the predicted zymogen cleavage site of rPC7 (5). Pro-rPC7 and rPC7 after either a 10-min pulse (Fig. 3) or 1-min pulse (not shown). It is thus plausible that zymogen cleavage is an event that occurs very early along the secretory pathway. This was indeed confirmed when a pulse of 30 min, followed by a chase of 60 min in the presence of BFA, showed that the ratio of the two bands was similar to that obtained in the absence of BFA (Fig. 3). Since the fungal metabolite BFA is known to disassemble the Golgi complex and produce fusion of the cis-, medial-, and trans-Golgi (but not the trans-Golgi network) with the endoplasmic reticulum (ER) (32), these results suggest that, like furin (32), PC1 (14), and PC5 (15), the processing of pro-rPC7 into rPC7 most likely occurs within the ER. Finally, in the presence of tunicamycin, a pulse of 30 min revealed the synthesis of two proteins with estimated masses of 83 and 75 kDa at a ratio of about 12:1. This suggests not only that rPC7 is N-glycosylated, but that, similar to PC1 (24), non-N-glycosylated rPC7 is more rapidly metabolized than non-N-glycosylated pro-rPC7.

In Vitro Properties of rPC7—The enzymatic activity of rPC7 was investigated using the fluorogenic substrate pERTKR-MCA (the best hydrolyzed fluorogenic substrate of those tested, see Table 1). Employing the ternary buffer system described under “Experimental Procedures,” we observed that the pH optima of both rPC7 and BTMD-rPC7 reside in the range of pH 6–7 (Fig. 5), a value similar to that of furin (11, 33). Interestingly, full-length rPC7 maintained a significantly greater percentage of its maximal activity than BTMD-rPC7 at nearly all of the pH values examined. Assay controls using membranes or media of VV-wild-type-infected BSC40 cells displayed <5% of the maximal rPC7 activity (results not shown). Moreover, addition of 10 mM EDTA completely abolished the activity of both forms.

A precise, direct determination of the Ca2+ requirement of

**Fig. 3. Biosynthesis of rPC7 in BSC40 cells.** VV:rPC7-infected BSC40 cells were pulse-labeled with [35S]Met for 10 or 30 min and then chased as indicated. Cell lysate and media samples were immunoprecipitated with the rPC7 antiserum and then resolved by SDS-PAGE, followed by autoradiography. The effects of 5 μg/ml of brefeldin A and 2.5 μg/ml of tunicamycin on rPC7 processing are depicted.

**Fig. 4. Microsequence analysis of pro-rPC7 and rPC7.** [H]Leu- or [3H]Met-labeled proteins from VV:rPC7-infected BSC40 cells were run on SDS-PAGE, eluted, and subjected to microsequence analysis as described under “Experimental Procedures.” The major and minor sequences obtained for the 101-kDa (upper panel) and the 89-kDa (lower panel) proteins are indicated below the figures. The cleavage sites are represented by the arrows.
rPC7 was not possible, since membrane preparations appear to contain high affinity Ca\textsuperscript{2+} binding sites. Thus, in experiments employing membranes to which no additional Ca\textsuperscript{2+} was added, the measured pERTKR-MCA hydrolytic activity was nearly 90% that of membranes to which 1 mM CaCl\textsubscript{2} had been added (not shown). Since preliminary attempts to wash out this residual Ca\textsuperscript{2+} were unsuccessful, an assay was carried out to determine how much EDTA would be required to abrogate the activity of rPC7. As shown in Fig. 6A, 1 mM EDTA added to the assay mixture was sufficient to nearly eliminate the hydrolysis of the MCA substrate by both rPC7 and BTMD-rPC7. We note that between 10 and 50 μM EDTA, the BTMD-rPC7 activity increased nearly 2-fold, whereas that of the membrane-bound rPC7 decreased by about 20%. Presumably, this is due to the presence of metal ions in the medium of BSC40 cells, which could inhibit BTMD-rPC7 activity as has been reported for PC1 and PC2 (34) as well as for furin (11, 33). Hence, the Ca\textsuperscript{2+} activation profile for rPC7 (Fig. 6B) was determined in the presence of 1 mM EDTA to begin the analysis at a minimal level of rPC7 enzymatic activity. The optimal Ca\textsuperscript{2+} concentration of the rPC7 and BTMD-rPC7 activities appears to be in the 1–2 mM range (Fig. 6B), again reminiscent of hfurin (11).

Tests using general protease inhibitors (Table II) indicate that rPC7's inhibitory profile is very similar to that of hfurin (11, 33). The only responses to serine protease inhibitors included modest inhibitory effects by 4-amidinophenylmethanesulfonyl fluoride and 1-chloro-3-tosylamido-7-amino-2-heptanone, which were probably dose-independent (not shown). Cysteine protease inhibitors and most mixed serine/cysteine protease inhibitors had no significant effects. An exception was antipain, which is a good inhibitor of ykexin (Table II) and is also known to slightly inhibit hfurin at 1 mM (33). As expected, the cation chelators EDTA, 1,10-phenanthroline, and, in particular, EGTA, were inhibitory. Thus, confirming that these enzymatic activities are indeed Ca\textsuperscript{2+}-dependent. Similarly, the strong inhibitory effects of oxidizing metals such as Zn\textsuperscript{2+} and Cu\textsuperscript{2+}, probably reacting with a metal-sensitive residue, have been previously described for PCs (33–35). Finally, a differential effect of reducing agents was observed. Thus, whereas 10 mM 2-mercaptoethanol, dithiothreitol, and glutathione enhanced the activity of rPC7, dithiothreitol inhibited BCRD-hfurin and ykexin, and glutathione activated ykexin but inhibited hfurin.

**Cleavage Profile of Fluorogenic Substrates**—The substrate specificity of rPC7 was examined and compared with those of BCRD-hfurin and ykexin using a series of synthetic fluorogenic MCA peptides of the general sequence ((R/K)\textsubscript{XX}R)-MCA. As indicated in Table III, the substrate best hydrolyzed by either rPC7 or ykexin during a 1-h reaction is the pentapeptide pERTKR-MCA. By comparison, a slightly better substrate for hfurin is RKKR-MCA, similar to findings reported using HIV gp160-related peptides (36). Interestingly, while cleavage of the model substrate RFAR-MCA is best afforded by hfurin, we note that all three enzymes can cleave to some degree at this monobasic site. The data also reveal that when the P-3 position is occupied by the acidic Glu residue (REKR-MCA), both hfurin and rPC7 do not cleave it very efficiently compared with ykexin. Also, using the RKKR-MCA substrate where the P-3 position is occupied by Lys, hfurin is more sensitive to this positively charged residue than rPC7 (compare REKR-MCA with RKKR-MCA).
and RSKR-MCA versus RKKR-MCA). Comparison of RSKR-MCA to RSKR-MCA shows that Arg at P-4 is much preferred as compared with Lys by both rPC7 and hfurin. Notably, ykexin tolerates Lys at P-4 and Glu at P-3 much better than either rPC7 or hfurin. Although the octapeptide YEKRKSR-MCA, representing the junction between the pro-segment of mouse PC1 and its catalytic subunit (37), contains the RSKR-MCA sequence, it is more poorly cleaved than the tetrapeptide. Finally, although X/KR/R-AMC tripeptides are well cleaved by ykexin (38), they were very poorly processed (<5% of the pERTKR-MCA hydrolysis) by either rPC7 or hfurin (not shown).

We next examined the cleavage kinetics of pERTKR-MCA by rPC7 and BTMD-rPC7. As depicted in Fig. 7, a representative experiment, the concentration dependence of the cleavage of this substrate is different for these two enzyme forms. Averaged values (three separate determinations) of the apparent $K_m$ values ($K_m$ (app)) of rPC7 and BTMD-rPC7, as well as of BCRD-hfurin and ykexin, for the pERTKR-MCA substrate are presented in Table IV. Thus, the $K_m$ (app) of BCRD-hfurin for this peptide is ~5 μM, as has been reported (26, 33). Although for ykexin the $K_m$ (app) is nearly 4-fold higher than that of hfurin, those of rPC7 and BTMD-rPC7 are approximately 14- and 28-fold higher, respectively. This suggests that rPC7 has either a relatively poor affinity for the above pentapeptide structure or a low catalytic (turnover) rate.

**Table II**

Effects of proteinase inhibitors on BCRD-hfurin, rPC7, and ykexin activity

| Inhibitor               | Control | APMPSF | PMSF | TLCK | TPCK | Appinin | α1-Antitrypsin | Soybean trypsin inhibitor | 3,4-Dichloroisocoumarin | Soybean trypsin inhibitor | CuSO4 | ZnSO4 | Glutathione | Dithiothreitol | 2-Mercaptoethanol | EGTA | EDTA | 1,10-Phenanthroline | Pepstatin | Leupeptin | Chymostatin | Antipain | Cystatin | E-64 | Iodoacetic acid | 3,4-Dichloroisocoumarin | Soybean trypsin inhibitor | CuSO4 |
|------------------------|---------|--------|------|------|------|---------|---------------|---------------------------|-------------------------|---------------------------|-------|-------|-------------|---------------|------------------|-------|------|------------------|----------|---------|-------------|---------|---------|-------|---------------|------------------------|--------------------------|-------|
| **inhibition**         | % control | 100.0 | 100.0 | 100.0 | 100.0 | % control | % control | % control | % control | % control | % control | % control | % control | % control | % control | % control | % control | % control | % control | % control | % control | % control | % control | % control | % control | % control | % control |
| **B**                  |         |       |      |      |      |         |               |              |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |
| **C**                  |         |       |      |      |      |         |               |              |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |

**Table III**

Comparative cleavage of fluorogenic synthetic peptidyl substrates by BCRD-hfurin, rPC7, and ykexin

| Substrate | Substrate hydrolysis | BCRD-hfurin | rPC7 | ykexin |
|-----------|----------------------|-------------|------|--------|
|           | % pERTKR-MCA hydrolysis |            |      |        |
| RFAR-MCA  | 40.6                 | 18.2        | 14.5 |
| RVRR-MCA  | 46.8                 | 8.6         | 12.2 |
| RKKR-MCA  | 13.7                 | 10.2        | 55.4 |
| RSKR-MCA  | 149.9                | 31.3        | 70.7 |
| KRKR-MCA  | 45.8                 | 41.8        | 70.7 |
| KSKR-MCA  | 5.9                  | 5.7         | 53.6 |
| pERTKR-MCA| 100.0                | 100.0       | 100.0|
| YEKRKSR-MCA| 18.2                 | 21.7        | 19.7 |

*Not determined.*

**Fig. 7.** Comparative kinetic analyses of pERTKR-MCA processing by rPC7 and BTMD-rPC7. Assays were carried out on enzyme preparations from VV:rPC7- and VV:BTMD-rPC7-infected BSC40 cells at optimal pH (6.5) and Ca$^{2+}$ (1 mM) conditions using increasing amounts of pERTKR-MCA. The curves in this representative experiment were fit to the data using nonlinear regression analysis (KaleidaGraph). The calculated $K_m$(app) values for this assay are 168.7 and 78.5 μM for rPC7 and BTMD-rPC7, respectively.

**Synthetic Peptide Digestion Profiles**—In an effort to determine a potential biological substrate of rPC7, three model synthetic peptides were examined. The choice of peptides was based on the colocalization of rPC7 with parathyroid hormone (PTH) in parathyroid glands (27), PC4 in testicular germ cells (5, 39), and EGF in submaxillary glands (40, 42). The peptides selected were as follows: 1) a 13-amino acid peptide spanning the prohormone cleavage site of human pro-PTH having the sequence KSVKKKR | SVSEIQK; 2) a 17-amino acid peptide containing the zymogen cleavage site of mouse PC4-proPC4 having the sequence YETLRRVKKR | SLVVPTD; and 3) a 16-amino acid human pro-EGF peptide having the sequence HLREDHHSYVR | NSDLS.

For both the pro-PTH and pro-PC4 peptides, a single cleavage by rPC7 (Fig. 8) or BCRD-hfurin (not shown) was observed within 10–20 min of reaction at the expected physiological site, which was ascertained by amino acid analysis of the products. However, for the pro-EGF peptide, no significant processing was detected, even after a 24-h incubation. Fig. 9 depicts the relative digestion of the pro-PTH (Fig. 9A) and pro-PC4 (Fig. 9B) peptides as a function of their concentration by either BCRD-hfurin, rPC7, or BTMD-rPC7. Based on these data, kinetic constants for peptide processing were calculated and compared with those of the pERTKR-MCA substrate (Table V). The $K_m$(app) and $V_{max}$ values of pro-PTH peptide processing are 1.5 μM and 1.8 μM/h for BCRD-hfurin; 11.9 μM and 30.4 μM/h for rPC7; and 5.5 μM and 28.3 μM/h for BTMD-rPC7. Thus, BCRD-hfurin has an 8- or 4-fold lower $K_m$(app) than either rPC7 or BTMD-rPC7 for this peptide. Moreover, it took at least 10 times more activity of the PC7 enzymes (as measured by hydrolysis at 100 μM pERTKR-MCA) to achieve the same extent of pro-PTH cleavage. The calculated $K_m$(app) and $V_{max}$ values of pro-PC4 processing are 1.4 μM and 13.0 μM/h for BCRD-hfurin, 2.2 μM and 4.3 μM/h for rPC7, and 1.5 μM and 21.1 μM/h for BTMD-rPC7 (Table V), again using approximately equal amounts of enzymatic activity measured at 100 μM pERTKR-MCA. In contrast, neither furin nor PC7 processed the pro-EGF peptide.

Using the ratio of $V_{max}$/K$_m$(app), it is possible to compare the
cleavage efficiency of a given enzyme preparation for various substrates. This analysis (Table V) indicates that processing of the pro-PTH peptide by furin is nearly 9 times more efficient than that of the pro-PC4 peptide. In the case of rPC7, the processing efficiency of both of these peptides appears to be similar, whereas BTMD-rPC7 exhibits a 2.7-fold preference for the pro-PC4 peptide. Interestingly, in comparison to the larger pro-PTH and pro-PC4 peptides, the cleavage efficiency of the fluorogenic pentapeptide substrate pERTKR-MCA is at least 30-fold lower for furin (27) and at least 50-fold lower for PC7.

**DISCUSSION**

Processing sites compatible with the cleavage selectivity of the known PCs are found in a variety of precursors that include prohormones and neural propeptides, serum proteins, cell-surface receptors, viral envelope glycoproteins, and growth factors (9). This wide spectrum of substrates is presumably the basis for the existence of a family of PCs expressed in a tissue-specific manner (3, 42) of which PC7 is the most recently discovered member (5–8). Sequence alignments suggest that PC7 is an ancestral member of the family of mammalian PCs, as it is the one most closely related to yeast kexin (5, 42). A widely expressed enzyme, PC7's tissue distribution is reminiscent of that of the ubiquitously expressed furin (5–8, 42). Moreover, like furin, PC7 is detected at high levels in lymphoid associated tissues (5, 42), suggesting that this convertase may play an important role in the immune system. Interestingly, certain human lymphomas express a modified version of PC7 mRNA (6). Until now, there have been no reports on the enzymatic properties of PC7 or identification of its potential physiological substrates. In the present work, we investigated the cleavage specificity and kinetics of rPC7 toward selected substrates in vitro, along with the zymogen processing and characterization of full-length and truncated forms of this enzyme. Our findings clearly demonstrate that this enzyme can cleave

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**TABLE IV**

Comparative $K_m$ values for the cleavage of the fluorogenic peptidyl substrate pERTKR-MCA by BCRD-hfurin, rPC7, BTMD-rPC7, and ykexin

| Enzyme      | $K_m$ (app) | $\mu M$ |
|-------------|-------------|---------|
| BCRD-hfurin | 5.9 ± 1.5   |         |
| rPC7        | 164.0 ± 18.7|         |
| BTMD-rPC7   | 58.8 ± 12.7 |         |
| ykexin      | 25.3 ± 1.5  |         |

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**FIG. 8.** Processing of the pro-PTH and pro-PC4 peptides by rPC7. The 13-amino acid pro-PTH and 17-amino acid pro-PC4 peptides were digested with rPC7 for 10 or 20 min, respectively, and the products were separated and purified by RP-HPLC using a 5-mm analytical Ultrasphere C18 column (Beckman), as described under “Experimental Procedures.” The linear gradient of acetonitrile is depicted. The peptides contained within the indicated peaks were identified and quantitated by amino acid analysis. The unlabeled peaks represent non-peptidic material which could be detected (absorbance at 210 nm) even in the absence of injected peptides.

**FIG. 9.** A, comparative kinetic analyses of pro-PTH peptide processing by BCRD-hfurin, rPC7, and BTMD-rPC7. Preparations of soluble BCRD-hfurin and BTMD-rPC7 as well as rPC7 membranes were assayed as described under “Experimental Procedures” for 10 min using increasing amounts of the synthetic pro-PTH peptide. RP-HPLC analysis of the digestion reactions was carried out as described above. Peptide concentrations were quantitated by amino acid analysis. The curves were fit to the data using nonlinear regression analysis (KaleidaGraph). The calculated kinetic constants are indicated in Table V. B, comparative kinetic analyses of pro-PC4 peptide processing by BCRD-hfurin, rPC7, and BTMD-rPC7. Assays were carried out as described above except that for rPC7 and BTMD-rPC7 the reaction time was increased to 20 min. The deduced kinetic constants are indicated in Table V.
TABLE V
Comparative kinetic constants for the cleavage of the synthetic peptidyl substrates pERTKR-MCA, proPTH, and proPC4 by BCRD-hfurin, rPC7, and BTMD-rPC7

| Peptide | Enzyme       | $K_{m(app)}$ | $V_{max}$ | $V_{max}/K_{m(app)}$ |
|---------|--------------|-------------|-----------|---------------------|
|         | BCRD-hfurin  | μM          | μM/h⁻¹    | h⁻¹                 |
| pERTKR-MCA |             | 5.9         | 1.8       | 0.31                |
| pro-PTH  |              | 1.5         | 126       | 84.0                |
| pro-PC4  |              | 1.4         | 13        | 9.29                |
| rPC7     |              | 164.0       | 6.4       | 0.04                |
| pro-PTH  |              | 11.9        | 30.4      | 2.55                |
| pro-PC4  |              | 2.2         | 4.3       | 1.95                |
|         | BTMD-rPC7    |             |           |                     |
| pERTKR-MCA |              | 74.0        | 7.5       | 0.10                |
| pro-PTH  |              | 5.5         | 28.3      | 5.15                |
| pro-PC4  |              | 1.5         | 21.1      | 14.1                |

a KSVKKR ↓ SVSEINL.  b YETLRRVKK ↓ SLVVPDT.  

substrates at paired basic residues, thus providing the first functional evidence for its membership in the family of mammalian subtilisin/kexin-like serine proteinases.

Overexpression of rPC7 in the constitutively secreting epithelial BSC40 cell line was carried out with the vaccinia virus expression system, which has previously been used to study the molecular forms of other PCs (11–14, 16, 25, 46). Using an antisem developed against a peptide sequence located within the P-domain of rPC7 (Fig. 1), we were able to demonstrate that rPC7 is a membrane-associated protein that resists solubilization by either 0.1 M Na₂CO₃ or low concentrations (<5%) of Triton X-100. This supports the prediction from the cdna sequence (5) that rPC7 contains a transmembrane domain. Furthermore, both Western blot (Fig. 2) and enzymatic activity analyses (Table I) of the media of cells overexpressing rPC7 failed to demonstrate the presence of a soluble, shed form of rPC7. This is in marked contrast to either hfurin (28, 29) or ykexin (31, Table I) which, when overexpressed in BSC40 cells, evince significant levels of shed protein in the culture medium. Interestingly, attempts to produce soluble forms of rPC7 ending either one (having the stop codon at Tyr-Gly-Ser²⁷⁹) or eight (having the stop codon at Val-Asp-Ile³⁶⁵) (5) residues after the putative C-terminal border of the P-domain (i.e. Gly-Ser²⁷⁹) (9, 43, 44) resulted in an inactive enzyme that was noted the presence of a single Cys⁶⁰⁹ (see Fig. 1) within the predicted site (Fig. 4).

The data presented in Fig. 6 and Table II demonstrate that rPC7 is a Ca²⁺-dependent serine proteinase having an optimal Ca²⁺ requirement of 1–2 mM (Fig. 6). The membrane-bound form of this enzyme has a particularly broad pH optimum, with maximal activity occurring at pH 6–7 (Fig. 5). In contrast, the secreted mutant form, which lacks the transmembrane domain and the cytosolic tail, has a narrower pH range (albeit the same optimum of pH 6–7). While this deleted region may exert a modulatory effect on catalytic activity, as is the case for hfurin (46), it is also possible that the membrane association of rPC7 affects the interaction of this enzyme with its substrates. Taken together, the pH and Ca²⁺ optima of rPC7 resemble those of hfurin (11, 33) and ykexin (38), both of which have been shown to be active within the constitutive secretory pathway (47). In contrast, enzymes such as PC1 and PC2, which are known to be localized in secretory granules (48), have a minimal Ca²⁺ requirement of 1–2 mM and a pH optimum of 5–5.5 (23, 49). However, we also note that rPC7 exhibits 40–60% of its maximal activity at pH 5–5.5. Therefore, we cannot exclude the possibility that rPC7 may be functional within acidic compartments such as endosomes. Accurate determinations of the intracellular localization of rPC7 are required to define the organelle(s) in which the active enzyme is residing. Immunocytochemical staining of rPC7 in VV-infected BSC40 cells revealed primarily a peri- and para-nuclear localization pattern, suggesting that this newest member of the PC family may reside in the ER and Golgi apparatus (15).

The cleavage specificity of rPC7 was first examined using a series of fluorogenic peptidyl substrates (Table III). A comparative analysis of hydrolysis of these MCA-containing peptides by BCRD-hfurin, rPC7, and ykexin indicates that rPC7 displays a cleavage specificity mostly resembling that of furin. For example, rPC7, like hfurin (26), does not hydrolyze tripeptide MCA substrates (even those containing a dibasic motif; data not shown), whereas ykexin does (38). Similarly, the REKR-MCA peptide, which contains a negatively charged Glu residue in the P-3 position, is poorly cleaved by both BCRD-hfurin (36) and rPC7 but is reasonably well cleaved by ykexin (Table III).

With regard to hfurin (see Ref. 26) and ykexin, these findings are consistent with the proposed structures of the S3 enzymatic subsites of these enzymes based on homology modeling with subtilisin (50). Thus, in hfurin, the predicted S3 subsite contains a pair of negatively charged Glu residues (Glu¹²³ and Glu¹⁵⁰ in Ref. 50), whereas in ykexin, Glu¹⁵⁰ is replaced by an Ala residue, presumably resulting in a lower negative charge density. However, in rPC7, the replacement of Glu¹⁵⁰ by a Pro residue does not enhance this enzyme’s tolerance for substrates containing Glu at the P-3 position. It is therefore possible that the identity of the amino acid corresponding to Glu¹⁵⁰ in the predicted S3 subsite of PCs can influence their substrate selectivity. This hypothesis may also account for our observation that the peptide RKKR-MCA, which contains a positively charged Lys residue at P-3, is better cleaved than the REKR-MCA peptide by both hfurin and rPC7 (Ref. 26, Table III). Also, the presence of an uncharged residue at the P-3 position (e.g. Ser in RKKR-MCA) is well tolerated by all three enzymes (Table III). With respect to the P-4 residue, the cleavage specificity of rPC7 also resembles that of hfurin rather than ykexin. Substitution of Arg by Lys in this position nearly abolishes MCA-peptide hydrolysis by BCRD-hfurin and rPC7, yet only decreases it by ~50% for ykexin (Ref. 26, Table III). In other respects, the cleavage specificity of rPC7 more resembles that

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of ykexin than of hfurin. For example, compared with BCRD-hfurin, rPC7 and ykexin rather poorly process the monobasic sequence RFAR-MCA or the double-Arg sequence RVRR-MCA (Table III).

The influence of other enzymatic subsites (P-5–P-8) may be inferred from the comparative hydrolysis of the two largest MCA-peptidyl substrates by BCRD-hfurin, ykexin, and rPC7 (Table III). The pentapeptide pERTKR-MCA, which is the best cleaved of the fluorogenic substrates tested, contains a pyro-Glu residue at the P-5 position. In contrast, the modestly hydrolyzed octapeptide YEKERSKR-MCA, representing the zymogen activation site of mPC1 (14), contains a Glu residue at both the P-5 and P-7 positions.

The different cleavage rates of these two peptides by rPC7 may be due to the replacement of the Glu residue by pyro-Glu in the pentapeptide.

Upon further investigation, a more detailed kinetic analysis suggested that pERTKR-MCA is a poor substrate for rPC7 as compared with BCRD-hfurin and ykexin (Table IV). The $K_m$ of rPC7 for this peptide was nearly 28 times higher than that of BCRD-hfurin and 6.5-fold higher than that of ykexin. Smaller variations were observed for the soluble BTMD-rPC7 enzyme (Fig. 7 and Table IV). This difference between the membrane-anchored and soluble forms of rPC7 may be due to an effect of the C-terminal region of this enzyme on its interaction with substrates (cf. Ref. 46). An alternative explanation is that nonspecific substrate adsorption to the membranes of the rPC7 enzyme preparation could decrease its availability, resulting in artificially high $K_m$ values. However, addition of VV-wild type-infected BSC40 cell membranes to an assay using BCRD-hfurin had no effect on the activity of rPC7, indicating that nonspecific substrate adsorption is unlikely to be a significant factor.

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The third synthetic peptide examined, pro-EGF, consists of a monobasic P-1 Arg cleavage site along with a Tyr residue in the P-5 position and a pair of His residues in the P-5 and P-6 positions (HLREDDHHYYSVR↓NSD). As indicated in Table V, this synthetic peptide is clearly not a substrate for either hfurin or rPC7 in our assays. We mentioned above that the monobasic Arg-containing peptide RFAR-MCA is poorly cleaved by both rPC7 and furin (Table III). Also, cleavage studies of other monobasic substrates reported for furin indicate that it requires additional P-4 or P-6 basic (Arg or Lys) residues (52). Thus, it appears that monobasic sequences lacking these additional basic residues (52) are not good substrates for these enzymes and that His does not appear to substitute for Arg or Lys at these positions.

Investigations of the substrate cleavage specificity of rPC7 using various fluorogenic peptides and synthetic model peptides mimicking precursors colocalizing with this enzyme did not allow us to identify a PC7-specific candidate precursor that was not also well cleaved by hfurin. Previously, hfurin was shown to be more efficient than hPC1 in processing the same pro-PTH peptide used in this in vitro study, as well as in processing pro-PTH to PTH following coexpression of the appropriate VV construct in BSC40 cells (27). Similar coexpression experiments are currently underway to compare the ex vivo processing of pro-PTH to PTH by hfurin and rPC7. Given their colocalization with pro-PTH in the parathyroid gland (27) and their ability to correctly process a peptide containing the pro-PTH processing site, it will be of considerable interest to determine whether both furin and PC7 are responsible for pro-PTH maturation in vivo.

With regard to the data on the pro-PC4 peptide, the biological relevance of these findings is not clear. Although PC7 is expressed in testicular germ cells, partially colocalizing with PC4 (5), there are as yet no reports of one PC being responsible for the zymogen activation of another. It is thus likely that the zymogen activation of PC4 is autocatalytic, as has been shown for furin (28, 30) and PC1 (53). However, as a model substrate, the sequence of the pro-PC4 peptide (LLRRVKR↓SLV...) closely mimics the zymogen processing sites of both hfurin (AKRRTKR↓DVY...) (11, 51) and rPC7 (LLKRKRAK↓SII...) (5). If, as mentioned, zymogen activation is autocatalytic, these sequences would necessarily function to some extent as biological substrates. As such, they warrant further investigation.

In general, the issue of which PC processes a given proprotein precursor remains largely unresolved. Considering the important biological functions of many PC substrates, it is possible that, for the organism's survival, evolution has chosen to ensure sufficient processing through the redundancy (3) of PCs. This could explain why the majority of tissues express both furin and PC7 (5, 42). On the other hand, although the intracellular localization of furin and PC7 appears to overlap to some degree, the exact intracellular trafficking of PC7 has not yet been described. It is possible that these two enzymes are active in mutually exclusive environments or that their activity is modulated by as yet unidentified cofactors. Moreover, the co-regulation of cognate PCs and their substrates (36, 54) favors the notion of a physiological coupling between each PC and particular substrates. Hence, variations in the level of cellular expression of a PC or its substrate could significantly influence the nature and extent of precursor processing (8). For example, PC7 is particularly abundant in certain activatable immune cells such as CD4+ T-lymphocytes (5, 42), suggesting that this enzyme may play an important role in processing precursors (e.g. the tumor necrosis factor receptor (55), integrins (56), and retroviral surface glycoproteins such as HIV gp160 (46)) expressed in either resting or activated immune cells. Taken together, these points illustrate the complexity of substrate recognition and cleavage by PCs and emphasize the need for multifaceted approaches to identify the cognate enzyme(s) responsible for the processing of precursor substrates under physiological conditions.

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