We have previously described the expression of the human proprotein convertase furin or paired basic amino acid-cleaving enzyme in mice transgenic for paired basic amino acid-cleaving enzyme and human Protein C (HPC). Here we show 100-fold or higher expression of furin in the mammary gland, compared with endogenous furin. Furin and recombinant HPC were detected in the same regions of the mammary gland and regulated similar to the endogenous whey acidic protein. In addition to the expected intracellular localization, furin was secreted into the milk as an 80-kDa form lacking the transmembrane and cytoplasmic domains. Furin present at levels of up to 40,000 units/ml milk cleaved the t-butoxycarbonyl-RVRR-AMC substrate with a $k_m$ of 32 $\mu$m, and processed the recombinant HPC precursor at the appropriate sites. Surprisingly, the expression of an active protease was not toxic to the mammary gland. This is a rare example of an animal model secreting active truncated forms of a processing endoprotease into a bodily fluid.

Furin or paired basic amino acid cleaving enzyme (PACE) is a Ca$^{2+}$-dependent serine protease that processes proteins in the constitutive secretory pathway (1). Furin belongs to a family of subtilisin-like proprotein convertases including kexin (EC 3.4.21.61), PC1/3, PC2, PC4, PC5/6, PACE4 (reviewed in Ref. 1), and PC7/PC8 or lymphoma proprotein convertase (2–5), that play a role in the maturation of proproteins by cleavage at dibasic or tetra-basic sites (6). Unlike PC1/3, PC2, and PC4, which are restricted to endocrine, neural, or testicular germ cells and oocytes, respectively, the furin gene is expressed in most tissues and cells examined (1, 7).

cDNAs cloned from several species show furin to contain prepro-, catalytic and middle domains, a cysteine-rich region, transmembrane anchor, and cytoplasmic domain (1). Furin is activated by autocatalytic cleavage of its propeptide, presumably at the -Lys-Arg-Arg-Thr-Lys-Arg$^1$. 1 site (1, 8). A membrane-associated protein, it is found mainly in the trans-Golgi network (TGN) co-localized with the TGN 38 marker protein (9). Some amount of furin recycles between the cell surface and the TGN (9) in endosome-like structures (10). So far furin has not been detected in bodily fluids like blood, nor has the effect of its expression on cell and organ development been studied in transgenic animals.

Transgenic animals secreting recombinant proteins into milk, blood, urine, or saliva (11) have been generated and differences in recombinant protein modification compared with the human counterpart observed (12). In a pioneering attempt to engineer the post-translational capacity of the mouse mammary gland, we reported enhanced proteolytic maturation of the recombinant human Protein C (rHPC) precursor upon co-expression with furin (13). However, the effect of increased furin concentration on mammary gland development could not be predicted, nor its’ intra- and extracellular distribution. Here we demonstrate localization of two heterologous proteins with the endogenous whey acidic protein (WAP) in the mouse mammary gland and secretion of active truncated furin into milk.

EXPERIMENTAL PROCEDURES

Transgene Construction and Generation of Transgenic Animals—The WAP/PACE construct comprising the 2.47-kb human furin cDNA and 74-base pair 3′–untranslated sequences, the four lines of CD-1 mice transgenic for WAP/HPC (12) and WAP/PACE expressing both transgenes in the mammary gland have been described (13).

Isolation and Northern Blotting of RNA—RNA was isolated from tissues of primiparous, F3 generation transgenic and control females on day 10 of lactation using RNAzol (Molecular Research Center). 15$\mu$g of total RNA was fractionated on formaldehyde, 1.2% agarose gels along with RNA markers (Life Technologies) and transferred to GeneScreen Plus membranes (DuPont NEN). A radiolabeled 2.5-kb human PACE cDNA was used to detect mouse furin as they share 94% amino acid homology (14). Blots were hybridized under low stringency conditions, annealing at 62 °C and low stringency washing at 55 °C. Data were corrected for sample loading by ethidium bromide staining and 18$S$ rRNA detection.

Immunohistochemical Detection of Recombinant Proteins—Paraffin-embedded sections of mammary gland from mid-lactation bigenic mice were stained with hematoxylin/eosin or immunostained. Endogenous peroxidase activity was exhausted by immersion in 0.3% hydrogen peroxide for 30 min. Sections were preincubated in 1% bovine serum albumin in phosphate-buffered saline (PBS) with 10% rabbit, horse, or goat serum for the detection of rHPC, furin, or mWAP, respectively. Sections were reacted with a 1:400 dilution of sheep anti-HPC antibody (Affinity Biologicals), a 1:5 dilution of MON 148 antibody (gift of Dr. Van de Ven; Ref. 15) or a 1:800 dilution of rabbit anti-mWAP antibody (gift of Dr. Hennighausen), and developed with biotinylated secondary antibodies in a 1:100 to 1:2000 dilution, an ABC Elite kit (Vector Laboratories), DAB substrate (Sigma), and Mayer’s hematoxylin as counterstain. Staining with secondary antibodies alone was negligible. Tissues of at least two animals from all four lines were analyzed.

Preparation of Milk and Western Blot Detection of Furin—Continuously bred mice of the F3 and F4 generations were milked between days 7 and 15 of lactation after intraperitoneal administration of 0.5 IU oxytocin (Sigma). Milk was diluted with 2 volumes of PBS, pH 7.4, containing 50 mM EDTA, centrifuged twice at 15,000 × g for 15 min at 4 °C, and stored at 80 °C. 20$\mu$g of milk proteins were separated under reducing conditions on 8–16% (Novex) or 10% SDS-polyacrylamide gels and silver stained, or Western blotted with 1:20 to 1:100 dilutions of the MON 139, 148, 150, or 152 anti-furin monoclonal antibodies (15). Horseradish peroxidase-conjugated goat anti-mouse IgG at 0.5 $\mu$g/ml was added and blots developed by enhanced chemiluminescence (Amersham). HPC was detected using the heavy chain-specific 8661 monoclonal antibody, or a sheep polyclonal antibody. Milk was fractionated essentially as described (16, 17). Briefly, 100 $\mu$L of milk containing sucrose at 5% (w/v) was underlaid beneath 1 ml of PBS and spun at 1,500 × g, for 30 min at 22 °C. A lower “skim milk” fraction was
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**RESULTS**

**Expression of Furin Transgene in Bigenic Mice**—Northern blot hybridization with the human probe under conditions of low stringency revealed a 4.2-kb furin transcript in control and transgenic mouse mammary glands, Fig. 1, corresponding to reports of a 4.5-kb RNA in mouse kidney (7) and a 4.0-kb RNA in other tissues (14). The signal obtained in the mammary gland was 5–10-fold lower than that in the liver, salivary gland, and brain (data not shown). The expected 2.5-kb human furin transcript was detected in bigenic mice at levels about 100-fold higher than endogenous furin.

**Detection of Recombinant Proteins in the Mammary Gland**—Histologic analyses of the mammary glands of HPC/PACE bigenic mice did not reveal differences in their gross morphology compared with control mice, Fig. 2. Transgenic tissues differed subtly, having less distended alveoli with larger epithelial cells, as in HPC transgenic mice (19). The distribution of recombinant proteins and endogenous WAP was compared by immunostaining of serial sections. rHPC was found in the secretory alveolar lumina and within epithelial cells, similar to rHPC and nWAP. The short arrow marks the same alveolus in different sections, while the boxed regions in C and D (100 ×) are enlarged in E and F (400 ×). The long arrow denotes discrete intracellular staining of furin.

**Purification of rHPC Processed by Secreted Furin**—Whole milk, from the HPC line 6.4, was incubated for 5 min at 37 °C with an equal volume of 100 μM peptide substrate Boc-RVRR-AMC (N-t-butoxycarbonyl-l-arginyl-l-valyl-l-arginyl-l-arginine-7-AMC; Bachem) in 100 mM HEPES, pH 7.5, containing 1 mM CaCl2 and 0.5% Triton X-100, in a 200-μl volume. Reactions were terminated by the addition of 100 μl of 15 mM EDTA. 7-Amino-4-methylcoumarin (AMC) liberated by cleavage was measured with a Perkin-Elmer LS-3 spectrofluorometer, 380 nm excitation, 460 nm emission. The blank sample consisted of substrate alone, while control milk values were negligible. For kinetic analysis, duplicate milk samples diluted 1:200 were incubated for 5 min at 37 °C with Boc-RVRR-AMC at concentrations ranging from 12.5 to 200 μM. Protease activity was assayed by incubating whole mouse milk from the HPC line 6.4 (12) in a 10:1 ratio with milk from HPC/PACE line C5.2, for 0–30 min at 37 °C. Milk was then diluted and processed with PBS, pH 7.4, with 50 mM EDTA to inhibit further furin activity.

**Characterization of Furin Secreted in Milk**—The furin-specific MON 148 antibody (15) detected a protein of approximately 80 kDa in bigenic mouse milk, which was absent in control milk (Fig. 3A). Higher levels were detected in lines C1.2, C2.2, and C5.2, than in C4.1. Trace polypeptides of approximately 150 kDa were also detected. A 141-kDa membrane-associated form of furin was recently reported in neural lobe secretory vesicles (20). MON 152 which is specific for human furin also detected the 80-kDa protein (Fig. 3B). The enzyme was not recognized by MON 150 and MON 139, indicating removal of the propeptide and C-terminal region, respec-
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The activity of secreted furin was determined by cleavage of a fluorogenic substrate. One unit of activity was defined as the amount of enzyme required to liberate 1 pmol of AMC from Boc-RVRR-AMC per min. Assays were performed in triplicate. $K_m$ values were calculated from Lineweaver-Burk plots of initial velocity measured at different substrate concentrations.

| Mouse line | Activity in milk (pmol/hr/ml) | Michaelis constant, $K_m$ (µM) |
|------------|-------------------------------|-------------------------------|
| C 1.2      | 21,973 ± 724                  | 32.26                         |
| C 2.2      | 27,470 ± 1,668                | 33.33                         |
| C 4.1      | 10,902 ± 359                  | 32.15                         |
| C 5.2      | 40,515 ± 706                  | 31.25                         |

**FIG. 4. Processing of rHPC and furin in the mammary gland.**

The WAP/HPC and WAP/PACE transgenes were coexpressed in the mouse mammary gland. Pro-furin composed of the propeptide (P), serine protease (SP), middle (M), Cys-rich (CYS), transmembrane (TM), and cytoplasmic (C) domains is activated by propeptide cleavage. Furin is localized to the TGN or undergoes C-terminal truncation and is secreted into the milk. Furin processes pro-Protein C inside cells to the mature form by removing the propeptide and the internal dipeptide. Processing may also continue in milk. The arrows denote known proprotein cleavage sites, while the dotted arrows indicate probable cleavage sites. The thin bars define the epitopes recognized by MON 139, 148, 150, and 152 antibodies, the darker bars the predicted antigenic regions (15).

**Table I**

**Activity of furin in bigenic mouse milk**

The activity of secreted furin was determined by cleavage of a fluorogenic substrate. One unit of activity was defined as the amount of enzyme required to liberate 1 pmol of AMC from Boc-RVRR-AMC per min. Assays were performed in triplicate. $K_m$ values were calculated from Lineweaver-Burk plots of initial velocity measured at different substrate concentrations.

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**DISCUSSION**

Normal cells express furin at low levels and the enzyme is ubiquitously distributed (7). Expression of furin in genetically engineered cells or animals at a great excess beyond the basal level deviates from the physiological norm and may be toxic. Toxicity of furin has been reported in transfected cells (24), probably due to broadened specificity. The furin gene was also up-regulated 5–10-fold in primary human non-small cell lung carcinomas (7), while PC7/PC8 has been implicated as a target for chromosomal translocation and deregulation (5). Various proteins involved in mammary growth and differentiation like insulin (25), insulin-like growth factor (6), insulin pro-receptor domains.

In HPC/PACE bigenic mice, rHPC was processed to the mature protein (13), unlike rHPC from HPC transgenic mice, which consisted mainly of the single chain precursor with propeptide (12). Incubation of milk from HPC mice alone did not alter the electrophoretic pattern of rHPC, but trace amounts of single chain present in HPC/PACE milk disappeared. rHPC purified from a mixture of HPC/PACE and HPC milk after incubation showed a marked reduction in the amount of precursor (Fig. 5B). Amino acid sequencing confirmed site-specific cleavage of the propeptide.

**Activity of Secreted Furin—Release of AMC from a fluorogenic substrate.**

Secreted furin cleaved the substrate with an average $K_m$ of 32.3 ± 0.85 µM, correlating with the reported values of 27 and 26 µM for furin secreted from VV:hFUR (23) and hFUR713t-infected BSC40 cells (18), respectively, and indicating similarity of the serine protease domains.

**FIG. 3. Western blot of furin secreted into milk.** Milk proteins were separated by SDS-PAGE in 8% gels under reducing conditions. A, blot of milk proteins from two control mice (CON) and from HPC/PACE transgenic mice from lines C1.2, C2.2, C4.1, and C5.2 was probed with a 1:100 dilution of MON 148. B, milk proteins from control and line C5.2 mice were Western blotted with the monoclonal antibodies MON 139, 148, 150, and 152 at a 1:20 dilution, as marked. C, furin was detected in different fractions such as the milk fat globule membrane (MFGM) supernatant (lane 1), MFGM pellet (lane 2), cream wash (lane 3), and skim milk (lane 4) from a C5.2 mouse, using a 1:100 dilution of MON 148 antibody. No furin was detected in control skim milk (lane 5). Positions of molecular weight markers are shown on the left. Mouse IgG (H) chains are detected by the secondary antibodies at 50 and 100 kDa.
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and mice, line C5.2 (sheep anti-HPC antibody. rHPC purified from the milk of HPC/PACE resolved by 8–16% SDS-PAGE. Western blots were probed with the mouse secondary antibody (lanes 1–6). The dot indicates a mouse protein recognized by the goat anti-mouse secondary antibody (lanes 1–6). B, rHPC was purified from a mixture of HPC/PACE and HPC mouse milk after incubation for 2 h at 37 °C (lanes 1, 3, 5, 7, and 9) to 30 min (lanes 2, 4, 6, 8, and 10). Milk was then diluted in PBS containing EDTA, defatted, and 20 μg of reduced proteins resolved on 8–16% gels. Western blot detection of rHPC was carried out using the 8861 monoclonal antibody (lanes 1–6) or the sheep polyclonal antibody (lanes 7–10). The dot indicates a mouse protein recognized by the goat anti-mouse secondary antibody (lanes 1–6). B, rHPC was purified from a mixture of HPC/PACE and HPC mouse milk after incubation for 2 h at 37 °C, by immunoadfinity chromatography using the 8861 antibody and resolved by 8–16% SDS-PAGE. Western blots were probed with the sheep anti-HPC antibody. rHPC purified from the milk of HPC/PACE mice, line C5.2 (lanes 1 and 2), from HPC transgenic mice (lanes 3 and 4), and from the mixture of HPC and HPC/PACE mouse milk (lanes 5 and 6), plasma-derived HPC (lane 7). SC, single chain; HC, heavy chain; LC, light chain. α-, β-, γ-HC represent HC glycoforms.

transforming growth factor-β1 (27), and parathyroid hormone-related protein (22) are also processed by furin. Stromelysin-3, a metallopeptase involved in mammary tissue remodeling, was activated by furin in cells and in solution (29). Thus, undesirable effects following the expression of an additional protease could not be excluded. We show here that although transgene transcripts were highly expressed, mammary gland gross morphology was similar in normal and transgenic animals. Mammary development was not impaired, nor were tumors observed in mice from any line for up to 2 years of age and over several generations, implying that increased furin expression in mammary epithelial cells was not associated with transformation. Our results also do not implicate secreted furin in tissue remodeling.

Two mechanisms could possibly explain our data. First, large amounts of the specific substrate, rHPC, may limit access of intracellular enzymes in milk. It will not be necessary to process incompletely modified protein could be directed to the circulation. We had expected furin to be shed from the apical plasma membrane of mammary cells in fat globules, but did not detect full-length protein in the MFGM fraction. Unlike the cystic fibrosis transductance regulator protein found predominantly in the milk fat of transgenic mice (19), this was not the main route of furin secretion. Finding truncated furin in all fractions of milk suggested secretion along with the milk proteins. Propeptide removal probably occurred in the endoplasmic reticulum, followed by targeting to the TGN in the presence of the cytoplasmic tail (9, 33). Cleavage of the acidic signal crucial for TGN retention then resulted in secretion (28, 29), implying that C-terminal processing of furin occurs in normal, untransformed mammalian cells.

The activity of secreted furin provided further evidence of propeptide processing, as propeptide removal was necessary for enzyme activation (9) and the presence of propeptide on a furin mutant led to loss of activity (36). In milk up to 40,000 units/ml active furin which functioned as a protease in the maturation of the rHPC precursor was detected, unlike the PACE-Sol mutant which was unable to process vWF in the medium of COS-1 cells (37). We therefore conclude that both the intracellular and truncated extracellular forms are active and process the rHPC precursor in epithelial cells as well as in milk (Fig. 4). Similarly, Prieto et al. (1995) described the presence of active human α1,2-fucosyltransferase in the milk of mice (38). Thus, it is possible to produce significant amounts of secreted forms of intracellular enzymes in milk.

Our results should encourage others to augment the processing of endogenous and heterologous proteins in transgenic animals. It will not be necessary to process incompletely modified recombinant proteins in vitro, instead this can be done simultaneously in vivo. The intra- and extracellular presence of furin allows for multiple interactions with its substrate, thus removing temporal and spatial constraints on proprotein processing and highlighting further advantages of protein production in transgenic animal bioreactors.

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