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Expression and Transient Nuclear Translocation of Protein Convertase 1 (PC1) During Mouse Preimplantation Embryonic Development

by

Carly St. Germain

A thesis submitted to

the Faculty of Graduate and Postdoctoral Studies

in partial fulfillment of the requirements for the degree of Master of Science

Department of Biochemistry, Microbiology, and Immunology,

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ABSTRACT

Preimplantation embryos express a number of hormones, neuropeptides and membrane receptors known to derive from proteolytic activation of their precursors by proprotein convertases (PCs). The goal of this study was to determine the pattern of expression of the neuroendocrine protein convertase 1 (PC1) in mouse preimplantation embryos. Previous data have shown that PC1 transcripts are detectable by reverse transcription-polymerase chain reaction in unfertilized and fertilized eggs as well as at all stages of preimplantation embryonic development (Croissandeau and Mbikay, unpublished data). In this study, we show by immunoblotting that the zymogen and mature forms of PC1 are present at these stages. Using immunofluorescence laser confocal microscopy, we have examined its subcellular location: PC1-specific staining was observed throughout the cytoplasm of unfertilized eggs. After fertilization, surprisingly, the staining was transiently concentrated in pronuclei. It relocated to the cytoplasm at post-zygotic stages and was particularly strong at junctions between blastomeres. The nuclear translocation of PC1 in fertilized eggs is probably mediated by its prodomain. Indeed, when transfected in human colon carcinoma LoVo cells, a mutant proPC1 incapable of cleaving off its prodomain accumulated in the nucleus. Furthermore, when N-terminally fused to a green fluorescent protein, this domain was able to direct the reporter protein to the nucleus of these cells. Collectively, these data suggest that PC1 is a potential convertase for precursor proteins in preimplantation embryos. They also raise the possibility of a nuclear function for this enzyme during zygote formation.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
Arg	arginine
Asp	aspartic acid
Asn	asparagine
BSA	bovine serum albumin
CCK	cholecystokinin
Chip	chromatic immunoprecipitation
Chr	chromosome
CPE	carboxypeptidase E
CMV	cytomegalovirus
CNS	central nervous system
CRR	cysteine-rich region
Cys	cystine
DAPI	4′,6-diamidino-2-phenylindole
DNA	deoxy-ribose nucleic acid
DTT	dithiothreitol
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EFLP	edge filter long-pass
ER	endoplasmic reticulum

FBS	fetal bovine serum
FSH	follicle-stimulating hormone
GAPDH	glyceraldehyde phosphate dehydrogenase
GHRH	growth hormone-releasing hormone
GnRH	gonadotropin-releasing hormone
GLP	glucagon-like peptide
GPC-3	glypican
hCG	human chorionic gonadotropin
HA	hemagglutinin
HB	homogenization buffer
His	histidine
HIV	human immunodeficiency virus
hRenin	human renin
HRP	horseradish peroxidase
hvWF	human pro-von Willebrand factor
IGF	insulin-like growth factor
IU	international units
KO	knockout
Lys	lysine
LPH	lipotropic pituitary hormone
mRNA	messenger ribonucleic acid
β-MSH	beta-melanocyte-stimulating hormone
NIDDM	non-insulin dependent diabetes mellitus

β–NGF	beta-nerve growth factor
NLS	nuclear localization sequence
PACAP	pituitary adenylate cyclase-activating protein
PAM	peptidyglycine-alpha-amidating-monooxygenase
PBS	phosphate buffered solution
PC	proprotein convertase
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PIC	protease inhibitor cocktail
PMSG	pregnant mare serum gonadotropin
POMC	proopiomelanocortin
Pro	proline
pro-ANF	proatrial natriuretic factor
pro-Alb	pro-albumin
pro-C3	pro-complement factor C3
proPC1	pro-proprotein convertase 1
PVA	polyvinyl alcohol
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
Ser	serine
SG	secretory granules
TBS	Tris-buffered saline
TCA	trichloroacetic acid

TE	trophectoderm
TGF-β	transforming growth factor beta
TGN	trans golgi network
UV	ultra violet
ZP	zona pellucida

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1. INTRODUCTION

1.1 Post-translational Proteolytic Modifications in the Secretory Pathway

The idea that inactive proteins exist as precursors that undergo proteolytic activation is a widely excepted biochemical concept. Precursor activation is usually accomplished through the proteolytic cleavage of a peptide bond on a target protein, resulting in the exposure or conformational change of its active site. The term zymogen is used to describe the protein in its inactive form prior to enzymatic activation. Typical zymogens include hormones, digestive enzymes, vasoactive products, blood coagulation factors, complement system factors, toxins, and growth factors (Neurath and Walsh, 1976).

There are two major streams of activation of precursor proteins (Steiner et al., 1980b). The first group includes precursor proteins, typically secretory proteins and hormones, which are processed within the cells prior to exocytosis. The second group involves proteins which are enzymatically activated after secretion by either a single cleavage or by a series of cleavages in a cascade of biochemical responses. Proteins in the first group follow the secretory pathway. They are biosynthesized in the endoplasmic reticulum (ER) where they loose the signal peptide that had targeted them to the secretory pathway; they undergo additional proteolytic cleavages as they traverse through the Golgi apparatus to the golgi network (TGN); then, the processed products are either kept in storage vesicles until a stimulus induces their secretion (regulated secretion) or they are immediately transported outside the cell (constitutive secretion) (Figure 1).

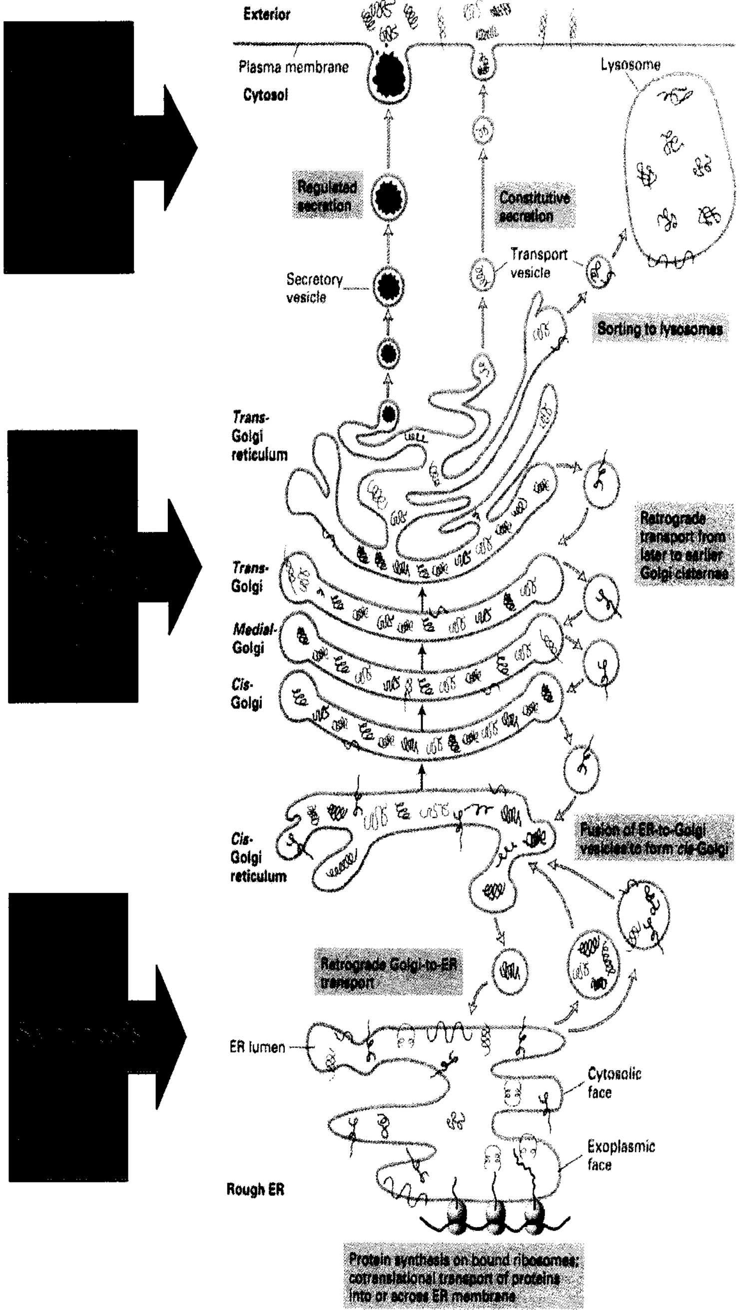
1.2 The Prohormone Theory

The idea that hormones existed as precursor forms which underwent processing was first introduced in 1967 following studies in two independent laboratories (Chretien and Li, 1967; Steiner et al., 1967). Using pulse-chase studies, Steiner et al. demonstrated that insulin derives from a larger precursor protein which they named proinsulin (Steiner et al., 1967). During chemical sequencing of pituitary peptides, Chrétien et al. (Chretien and Li, 1967) observed that β-melanocyte-stimulating hormone (β-MSH) was contained within the sequence of β-lipotropic hormone (β-LPH). They also observed that the β-MSH sequence within the β-LPH sequence was flanked by pairs of basic residues.

These seminal studies formed the foundation of the prohormone theory. According to this theory, peptide hormones derive from limited endoproteolysis of larger inactive precursors after pairs of basic residues (Bradbury et al., 1976; Chretien et al., 1980; Gainer et al., 1985; Lazure et al., 1983; Steiner et al., 1980a). This theory was confirmed over the years for a wide variety of bioactive peptides as their precursors were elucidated by cDNA cloning and sequencing (Steiner et al., 1980a). The dibasic maturation motif was also observed in most of these precursors, initiating the search for the endoproteinases responsible for these cleavages. It was also noted that cleavages after pairs of basic residues is generally followed by removal of the carboxyl terminal basic residues by carboxypeptidase E (CPE) and modification of C-terminal glycines into amide group by peptidylglycine-α-amidating-monooxygenase (PAM) (Eipper et al., 1992; Gainer et al., 1985).

FIGURE 1. *Proteolytic Processing in the Secretory Pathway.* Ribosomes synthesizing proteins bearing an ER signal sequence become bound to the rough ER. As translation is completed on the ER, the polypeptide chains are inserted in to the ER membrane or cross into the lumen. Some proteins (e.g., rough ER enzymes or structural proteins) remain resident in the ER. The remainder move into transport vesicles that fuse together to new cis-Golgi vesicles. Each cis-Golgi cisterna, with its protein contents, physically move from the cis to the trans face of the Golgi stack (red arrows). As this cisternal progression occurs, many luminal and membrane proteins undergo modification, primarily to attached oligosaccharide chains. Some proteins remain in the trans-Golgi cisternae, while others move via small vesicles to the cell surface or to lysosomes. In certain cell types (e.g. nerve cells and pancreatic acinar cells), some soluble proteins are stored in secretory vesicles and are released only after the cell receives an appropriate neural or hormonal signal (regulated secretion). In all cells, certain proteins move the cell surface in transport vesicles and are secreted continuously (constitutive secretion). Like soluble proteins, integral membrane protein move via transport vesicles from the rough ER to the cis-Golgi and then on to their final destination. The orientation of a membrane protein, established when it is inserted into the ER membrane, is retained during all the sorting steps: Some segments always face the cytosol; others always face the exoplasmic space (i.e., the lumen of the ER, Golgi cisternae, and vesicles or the cell exterior). Retrograde movement via small transport vesicles retrieves ER proteins that migrate to the cis-Golgi and returns them to the ER. Similarly, cis- or medial- Golgi proteins that migrate to a later compartment are retrieved by small retrograde transport vesicles (Figure 17-13, Lodish et.al, 2000).

The Secretory Pathway: Protein sorting and synthesis



Molecular Cell Biology. Lodish et al. Figure 17-13

1.3 Discovery of Proprotein Convertases

The search for the enzymes responsible for cleavages at basic residues lasted for nearly 30 years. The prospects for their identification improved with the cloning of the KEX2 gene which specified a subtilisin-like proteinase (kexin) capable of cleaving yeast pheromones as well as mammalian precursors after pairs of basic residues (Bathurst et al., 1987; Julius et al., 1984; Thomas et al., 1988). In 1989, Fuller et al. (Fuller et al., 1989), while searching the GenBank database for sequences homologous to Kex2, identified a protein showing significant homology to the yeast enzyme around the active residues of the catalytic domain. The protein named furin (**fes** **up**stream prote**in**) was thought to be a receptor (Roebroek et al., 1986b). Unbeknownst to them, the Belgian investigators who first cloned the furin mRNA had discovered the first mammalian proprotein convertase (PC).

Using degenerate primers based on sequence homology around the active site residues in the catalytic domains of kexin and furin, an American and a Canadian team independently amplified PC2 PCR DNA fragments from total cDNA from a human insulinoma and a mouse pituitary library, respectively. These fragments were used as probes to clone the full length cDNAs from which the sequence of the PC1 and PC2 were deduced (Seidah et al., 1991a; Smeekens et al., 1991; Smeekens and Steiner, 1990). PC1 and PC2 sequences exhibited high homology, not only to each other, but to kexin and furin as well. Expression of PC1 and PC2 mRNA were shown to be particularly concentrated in tissues known to be rich in hormones and neuropeptides such as the brain and the pituitary (Seidah et al., 1991a). More importantly, PC1 and PC2 were shown to cleave proopiomelanocortin (POMC) ex vivo, at distinct pairs of

basic residues, in a pattern known to occur in vivo, confirming for the first time that they were the long-sought prohormone convertases (Benjannet et al., 1991). Proinsulin processing by these convertases was demonstrated soon after (Smeekens et al., 1992).

In subsequent years, four more mammalian PCs were identified, namely PACE4 (Barr et al., 1991), PC4 (Nakayama et al., 1992; Seidah et al., 1992), PC5 (Lusson et al., 1993), also known as PC6 (Nakagawa et al., 1993), PC7 (Seidah et al., 1996), also known as PC8 (Bruzzaniti et al., 1996) or LPC (Meerabux et al., 1996). These enzymes were identified in many other phyla besides mammals, including molluscs (Gorham et al., 1996; Nagle et al., 1995a; Nagle et al., 1995b; Ouimet et al., 1993), amphibians (Braks et al., 1992; Gangnon et al., 1999; Gangnon et al., 2003; Vieau et al., 1998; Yaoi et al., 2003), arthropods (Cieplik et al., 1998; Mentrup et al., 1999; Roebroek et al., 1991; Smit et al., 1994) and nematodes (Gomez-Saladin et al., 1994; Kovaleva et al., 2002; Oliva et al., 2000; Poole et al., 2003).

1.4 Structure of Proprotein Convertases

The structural features of mammalian PCs have been described in several reviews (Brenner, 2003; Seidah and Chretien, 1999; Seidah and Prat, 2002; Steiner, 1998). All PCs are biosynthesized as multidomain glycoproteins. These domains are diagrammatically shown Figure 2. They include, successively: (i) a signal peptide sequence at the N-terminal which is cleaved off in the ER during early biosynthesis and functions to direct the precursor enzyme into the secretory pathway; (ii) a prodomain that is autocatalytically cleaved in the ER and functions as an intramolecular chaperone and inhibitor; (iii) a highly conserved catalytic domain which contains a catalytic triad (Asp, His, and Ser) and an Asn oxyanion hole characteristic of the serine family of

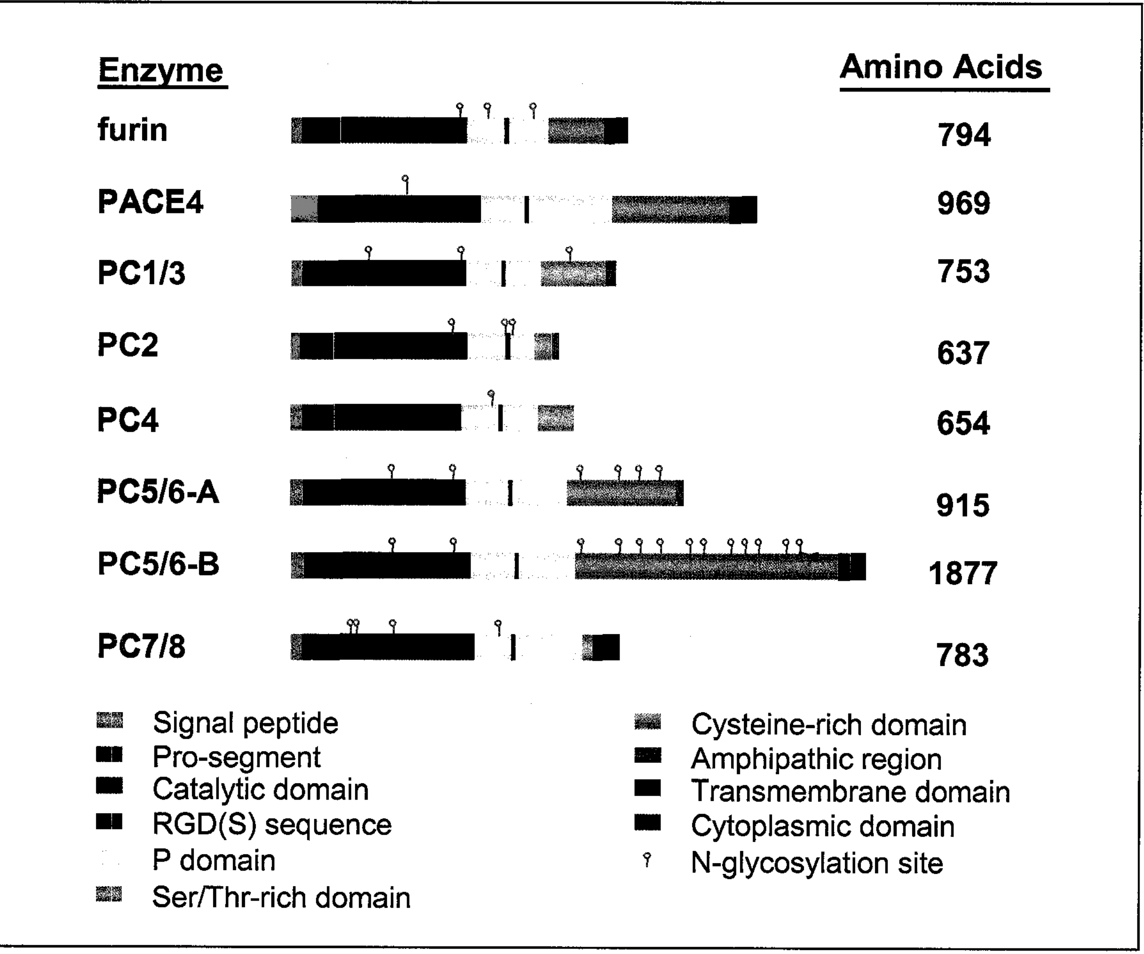
proteinases; (iv) a so-called P domain containing a conserved integrin-ligand RGD motif which is critical for enzymatic activity; (v) a C-terminal domain that is the least conserved region. In some PCs, this last domain contains a hydrophobic transmembrane domain followed by a cytoplasmic tail (furin, PACE4, PC5/6B and PC7/8) or a cysteine-rich region (CRR) in others (furin, PACE4 and PC6).

1.5 Proprotein Convertase Genes

Although mammalian PC genes vary in size and in exon number (Table 1), their exon-intron organization is highly conserved in the region specifying the prodomain to the P domain, in that introns interrupt the coding sequences at similar positions (Chan et al., 1992; Ftouhi et al., 1994; Goodge et al., 1998; Mbikay et al., 1994; Mentrup and Weidemann, 1999; Ohagi et al., 1992). This conservation suggests that PC genes may have arisen from a common ancestral gene.

The chromosomal location of PCs have been mapped in human and mouse genomes by in situ hybridization on metaphase chromosomes (Chr), Southern blotting analysis of panels of mouse-human hybrid cells, as well as linkage analysis (Bruzzaniti et al., 1996; Copeland et al., 1992; Kiefer et al., 1991; Mbikay et al., 1995; Meerabux et al., 1996; Seidah et al., 1996; Seidah et al., 1991a; Seidah et al., 1991b; van de Loo et al., 1996). The chromosomal loci are given in Table 1.

FIGURE 2. *Structure of Proprotein Convertases.* Diagrammatic representation of the structural domains of mammalian protein convertases. The domains are color-coded and explained in the legend below. The number of residues for each convertase is given to the right.



Enzyme
Amino Acids
furin
794
PACE4
969
PC1/3
753
PC2
637
PC4
654
PC5/6-A
915
PC5/6-B
1877
PC7/8
783
Signal peptide
Pro-segment
Catalytic domain
RGD(S) sequence
P domain
Ser/Thr-rich domain
Cysteine-rich domain
Amphipathic region
Transmembrane domain
Cytoplasmic domain
N-glycosylation site

TABLE 1. *Structure of Genes for Proprotein Convertases and their Companion Proteins**

Protein	Chromosomal Loci				Gene and mRNA Structure		
	Human		Mouse		Gene Size	Exon No	mRNA Isoforms
	Symbol	Chr	Symbol	Chr	kb		
PC1	*PCSK1*	5q	*Pcsk1*	13	42	14	5.1, 3.3, 3.0
PC2	*PCSK2*	20p	*Pcsk2*	2	256	12	4.8, 2.8
Furin	*PCSK3*	15q	*Pcsk3*	7	15	16	4.1
PC4	*PCSK4*	19q	*Pcsk5*	10	7	15	2.8, 1.9
PC5	*PCSK5*	9q	*Pcsk5*	19	301	38	7.5, 6.5, 3.3
PACE4	*PCSK6*	15q	*Pcsk6*	7	120	7	4.5, 3.9
PC7	*PCSK7*	11q	*Pcsk7*	9	25	17	3.5
CPE	*CPE*	4q	*Cpe*	8	111	9	2.5
SAAS	*PCSK1N*	Xp	*Pcsk1n*	X	5	7	1.0
7B2	*SGNE1*	15q	*Sgne1*	2	33	6	1.1

*The sizes of the genes were derived by calculating the distance in bases between the known 5' and 3' end sequences within a single contig. Most PC mRNAs are produced in multiple isoforms. Exon numbers are given for mouse encoding genes.

The locus symbols, *PCSK* in human and *Pcsk* in mouse, are acronyms for **p**roprotein **c**onvertase **s**ubtilisin-**k**exin-like. All PC genes are dispersed throughout the genome, except for the genes from furin and PACE4 which are less than 10 megabases apart on human Chr 15 (Kiefer et al., 1991) and on mouse Chr 7 (Mbikay et al., 1995). The close location of these two genes in both species suggests that the two genes may have arisen from a genomic duplication shortly before the branching away of the mouse and human evolutionary lines.

Most PCs are transcribed into multiple mRNA isoforms of different sizes (Seidah et al., 1994) (Table 1). These isoforms were generated through (i) alternate promoter use (Ayoubi et al., 1994); (ii) alternate exon splicing (Mbikay et al., 1994; Tsuji et al., 1997; Zhong et al., 1996); (iii) alternate use of polyadenylation signal regions (Ftouhi et al., 1994). These isoforms may be translated into a single protein (Ayoubi et al., 1994; Ftouhi et al., 1994) or into differing protein isoforms (Mbikay et al., 1994; Seidah et al., 1992; Tsuji et al., 1997). They are often found at differing levels in the same tissues but can also be tissue-specific (Seidah et al., 1994; Tsuji et al., 1994).

1.6 Tissue Distribution and Subcellular Localization of Proprotein Convertases

All tissues and cells contain multiple PCs, but in variable combinations and relative levels (Seidah et al., 1994; Seidah et al., 1996). Table 2 summarizes the tissue distribution of these enzymes. PC1 and PC2 are found primarily in neuronal and endocrine tissues. In situ hybridization analysis in the central nervous system (CNS) has shown extensive overlapping as well as differential regional abundance of PC1 and PC2 transcripts. PC1 transcripts are relatively more abundant in the supraoptic and

paraventricular nuclei of the hypothalamus, whereas those of PC2 are enriched in the superficial layers of the cortex, amygdala and the striatum of the hippocampus (Day et al., 1993; Schafer et al., 1993). In the pituitary, PC1 transcripts are more abundant in the anterior lobe and those of PC2 transcripts more so in the intermediate lobe (Marcinkiewicz et al., 1993; Seidah et al., 1991a).

PC4 transcripts are detectable by northern blot and in situ hybridization only in testicular germ cells, particularly in the spermatocytes and round spermatids (Nakayama et al., 1992; Seidah et al., 1992; Torii et al., 1993). Small amounts of PC4 transcripts are reportedly expressed in resident macrophage-like cells of the ovary (Tadros et al., 2001).

Furin, PACE4, PC5 and PC7 are wildly distributed. Furin mRNA is particularly abundant in the liver and the kidney (Roebroek et al., 1986a). PC5 mRNA is highly expressed in the gut, adrenal glands, ovaries and lungs (Lusson et al., 1993). PC7 transcripts are enriched in the thymus and the testis (Seidah et al., 1996). In the CNS, the pituitary and peripheral tissues, PC5/6 and PACE4 are distinctly distributed (Dong et al., 1995; Villeneuve et al., 1999; Zheng et al., 1997).

At the subcellular level, PCs are also differentially localized (Figure 3): PC1, PC2 and PC5B are stored in the secretory granules of neuroendocrine cells (De Bie et al., 1996; Itoh et al., 1996; Kirchmair et al., 1992; Kurabuchi and Tanaka, 2002; Malide et al., 1995; Marcinkiewicz et al., 1994; Muller et al., 1998; Tanaka et al., 1996; Uehara et al., 2001); furin, PC5B and PC7 and are found in the golgi and at the cell surface (De Bie et al., 1996; Molloy et al., 1994; van de Loo et al., 1997; van de Loo et al., 2000).

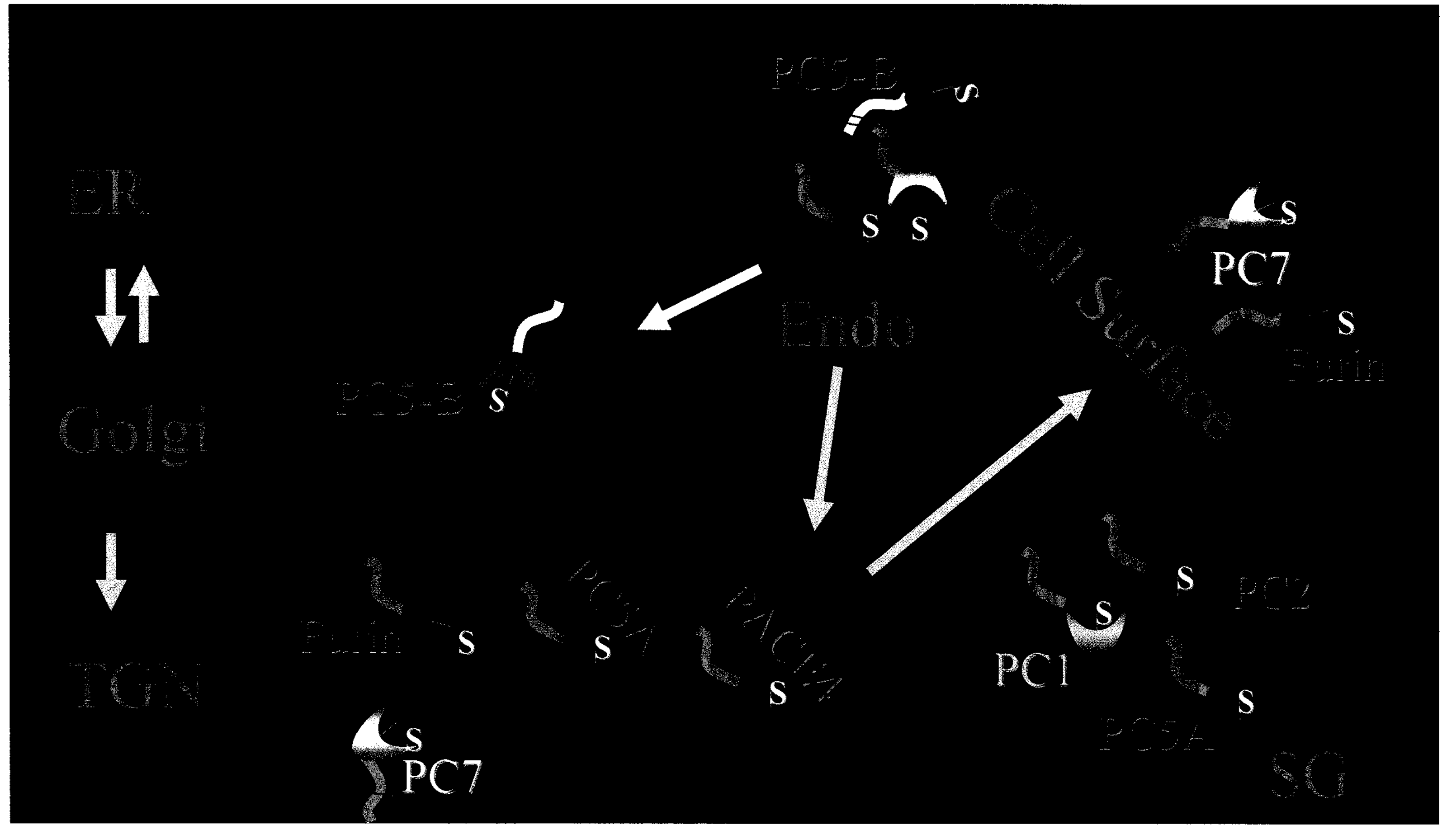
TABLE 2. *Tissue Distribution of Proprotein Convertases*

PC	Tissue Specificity	Major Expression Sites*
PC1	Neuroendocrine	CNS (PVN, SON), pituitary (AL)
PC2	Neuroendocrine	CNS (cortex, amygdala, striatum, hippocampus), pituitary (NIL)
PC4	gonadal	testicular germ cells
PC5	widespread	gut, adrenal glands, ovaries, lungs
PC7	ubiquitous	thymus, testes
Furin	ubiquitous	liver, kidney
PACE4	widespread	pituitary (AL), heart, kidney

*Based on Seidah et al., 1992 and Seidah et al., 1996

Abbreviations: AL, anterior lobe; CNS, central nervous system; NIL, neurointermediate lobe; PVN, paraventricular nucleus; SON, supraoptic nucleus

FIGURE 3. *Subcellular Localization of Proprotein Convertases.* Diagrammatical representation of the subcellular localization of PCs as they move through the compartments of the secretory pathway. PC1, PC2, and PC5A are stored in the secretory granules (SG) of neuroendocrine cells and PACE4 are located in the Trans Golgi Network (TGN). Furin, PC5B and PC7 are also found at the plasma membrane from where they can be transported back to the golgi and the TGN via endosomes (endo).



ER
Golgi
TGN
PC5-B
PC5-B
Endo
Cell Surface
PC7
Furin
Furin
PC7
PACE4
PC1
PC2
PC5A
SG

1.7 *Biosynthesis of Proprotein Convertases*

The biosynthesis and enzymatic activity of PCs have been extensively investigated and their common features have been described in details in several excellent reviews (Muller and Lindberg, 1999; Seidah and Chretien, 1999; Seidah and Prat, 2002; Steiner, 1998; Van de Ven et al., 1993). All PCs are biosynthesized in the ER as preproproteins. After co-translational removal of the signal peptide, they undergo a series of post-translational modifications as they travel through the various compartments of the secretory pathway to their subcellular destinations. One of the early modifications occurring in the ER is an autocatalytic cleavage after pairs of basic residues located between the prodomain and the catalytic domain. Based on studies conducted on furin (Anderson et al., 2002), it is believed that the prodomain remains attached in a complex with the molecule until a secondary autocatalytic cleavage occurs after a pair of basic residues located within the prodomain where the more acidic environment of the TGN induces their separation. The prodomain acts as an intramolecular chaperone that facilitates the proper folding of the zymogen and in a complex with the mature enzyme, it inhibits its enzymatic activity. More or less specific PC inhibitors have been designed based on propeptide sequences (Basak and Lazure, 2003; Boudreault et al., 1998a; Nour et al., 2003; Zhong et al., 1999). Other post-translational modifications effected on various PCs in the golgi compartments include glycosylation, sulfation, phosphorylation and palmitoylation (Benjannet et al., 1993; Jones et al., 1995; van de Loo et al., 2000). In post-TGN compartments, some PCs undergo further endoproteolytic truncations at the C-terminus. For PC1, these truncations lead to the production of a most active, yet unstable form of the enzyme

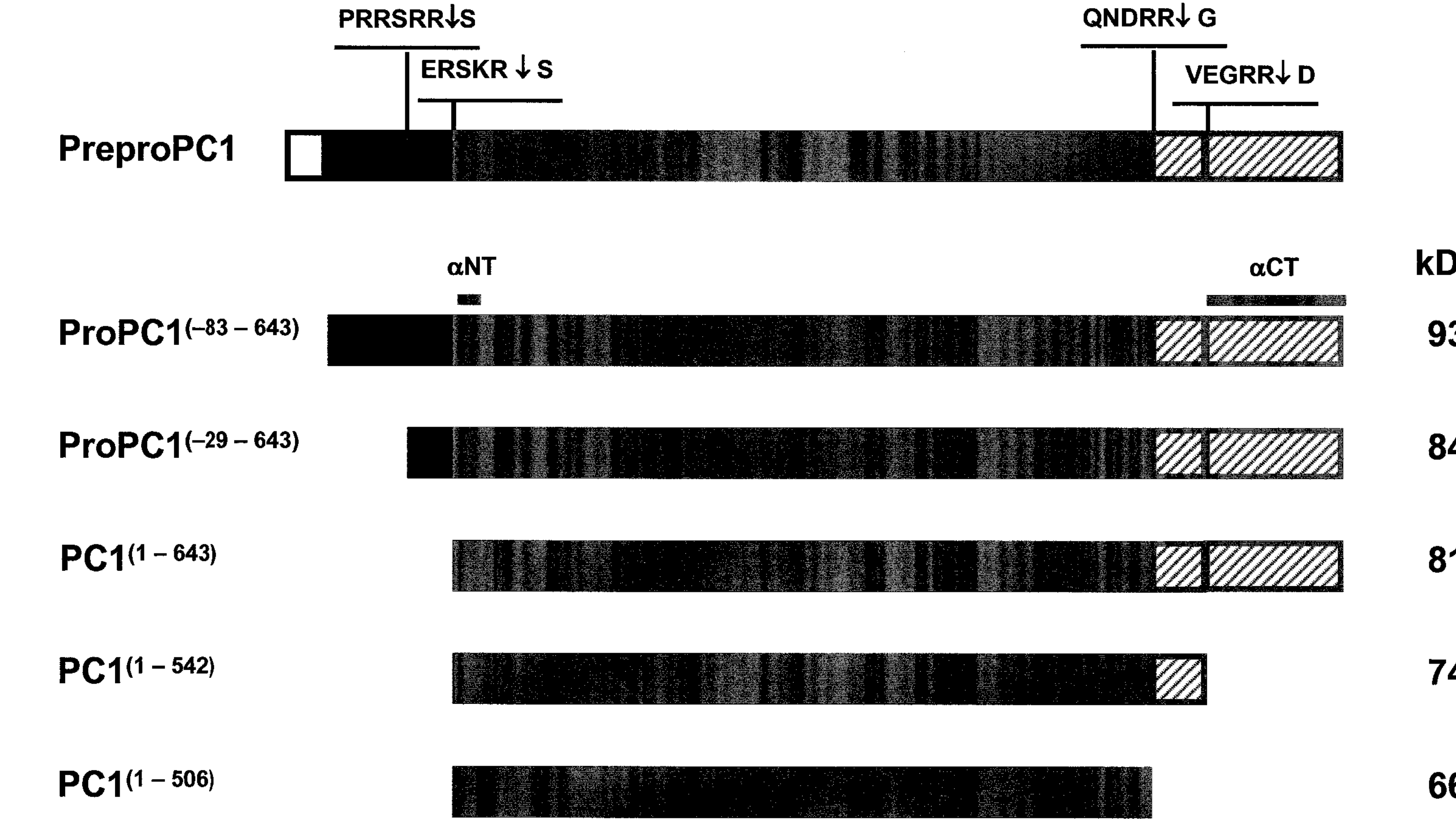
(Muller and Lindberg, 1999). The endoproteolytic modifications undergone by preproPC1 during biosynthesis are diagrammatically illustrated in Figure 4.

1.8. Enzymatic Activities of Proprotein Convertases

All PCs are calcium-dependent serine endoproteinases. They are all active at millimolar concentrations of Ca^{2+}. However, they vary in optimum Ca^{2+} concentration and optimum pH. For example, furin is most active at 0.2 mM Ca2+ and at near neutral pH (Molloy et al., 1992), whereas PC1 activity is highest at 4 mM Ca2+ and a more acidic pH of 5 to 6 (Boudreault et al., 1998b; Zhou and Lindberg, 1993; Zhou and Lindberg, 1994). These differential requirements determine in which cellular compartment each convertase is optimally active. Thus, furin may be active in the TGN where the calcium level is low and the pH is close to neutral whereas PC1 will be most active in the more acidic and calcium-enriched environment of neuroendocrine secretory granules (SG).

PCs cleave their substrates generally after pairs of basic residues, occasionally after single basic residues. The consensus recognition motif is **(Arg/Lys)-(X_n)-Arg↓** where n=0, 2, 4 or 6 and X is any amino acid except Cys and rarely Pro (Seidah and Chretien, 1999). Furin specifically requires an Arg at position 4 (P4) before the cleavage site. A His at P6 may make the site more specific for PC5 (Bergeron et al., 2003) and a Lys at P4 more specific for PC4 (Basak et al., 1999; Basak et al., 2004). Interestingly, not all sites fitting the consensus motif are cleaved by PCs.

FIGURE 4. *Endoproteolytic Processing of PreproPC1 during Biosynthesis.*The mouse PC1 gene is transcribed into two mRNA isoforms of 2.8 and 4.4 kb that are translated in the ER into a preproprotein of 753 amino acids. After early removal of the signal peptide by a signal peptidase, the resulting 93-kDa proPC1 is autocatalytically (enzymatically cleaves self) converted to an intermediate form and a mature form of 84 and 81 kDa by undergoing cleavages at pairs of basic residues within and after the prodomain, respectively. In the TGN and SG compartments the mature 81 kDa form undergoes further self-induced cleavage at its C-terminal to yield a more active form of 74 kDa. Additional C-terminal autocatalytic modification results in the final and most active form of 66 kDa (Muller and Lindberg, 1999). In these diagrammatic representations of PC1 isoforms, the signal peptide is represented as an open bar, the prodomain as a black bar, the catalytic and P domains as a single grey bar and the cleavable C-terminal domains as hatched bars. The sequences around the putative cleavage sites are shown above the bars. The regions recognized by the antibodies at the N and the C terminus of PC1 are shown above the proPC1 bar and marked as αNT and αCT, respectively. The apparent molecular weights of the various PC1 forms are indicated to the right of each bar.


PRRSRR↓S
ERSKR ↓ S
QNDRR↓ G
VEGRR↓ D
PreproPC1
αNT
αCT
kDa
ProPC1(−83 – 643)
93
ProPC1(−29 – 643)
84
PC1(1 – 643)
81
PC1(1 – 542)
74
PC1(1 – 506)
66

Clearly other yet unknown structural constraints on the substrates and the PCs determine which potential sites in a protein can be recognized by the enzymes. Recently a public algorithm called ProP was developed to predict PC cleavage sites (Duckert et al., 2004). It appears to be more sensitive for furin sites than for general PC sites.

The cellular activities of PCs may be modulated by co-resident proteins. Two such modulating proteins found in neuroendocrine cells are 7B2 and proSAAS. 7B2 acts as specific chaperone for PC2 and may be a transient inhibitor of its activity. It is produced as a precursor protein that gets cleaved to release a C-terminal inhibitory peptide. The structure and functions of 7B2 have been recently reviewed (Mbikay et al., 2001; Muller and Lindberg, 1999). ProSAAS is a specific inhibitor of PC1 activity (Fricker et al., 2000). It is the precursor to a number of peptides (Sayah et al., 2001). Its inhibitory activity is located in the C-terminal region (Qian et al., 2000) (Basak et al., 2001).

The cellular activities of PCs might be influenced by the efficiency of removal of the C-terminal basic residues exposed by PC cleavage. This removal is mediated by carboxypeptidases (CP) E and D (Dong et al., 1999; Fricker, 1988). It is believed that the multiple deficiencies of prohormone and proneuropeptide processing observed in a CPE deficient mouse are due to PC inhibition by the accumulation of basic residue-extended processing intermediates (Fricker and Leiter, 1999; Varlamov et al., 1997).

1.9 Proprotein Convertase Substrates

Generally, an enzyme-substrate relationship between a PC and a precursor protein is established by co-transduction of the enzyme and the potential substrate into a cell line using expression vectors followed by analysis of the precursor-derived smaller proteins. The first experiments conducted in the early nineties to this end established that furin could appropriately cleave human-β-nerve growth factor (Bresnahan et al., 1990), pro-von Willebrand factor (vWF) (van de Ven et al., 1990; Wise et al., 1990), pro-albumin and pro-complement factor C3 (Misumi et al., 1991). By far the most significant was the elegant study by Benjannet et al. in 1991 (Benjannet et al., 1991), demonstrating that PC1 and PC2 can cleave POMC in a pattern concordant with their distribution and relative levels in pituitary lobes.

Since these seminal studies, the number of confirmed PC substrates has increased exponentially. Table 3 shows the variety of classes of proproteins (with some representative members of each class given in parentheses) known to be cleaved by these endoproteinases. Because multiple PCs are found in all cells, a redundancy of their activities was anticipated. However, their differential subcellular localization suggested that they may play distinct processing roles. Both substrate redundancy and specificity have been experimentally demonstrated.

Because of their characteristic expression in neuroendocrine and endocrine tissues, PC1 and PC2 are expectedly involved in the processing of prohormones and proneuropeptides. Representative precursors processed by these two enzymes include POMC (Benjannet et al., 1991; Zhou et al., 1993), proinsulin (Smeekens et al., 1992) and proglucagon (Dhanvantari et al., 1996). The two enzymes sometimes act on these

substrates sequentially: PC1, which is active in earlier compartments, cleaves the precursor into intermediates; PC2, which is activated in later compartments then cleaves the intermediates into final products. The proportion of intermediate and final products depends on the relative abundance of the two enzymes.

PC5, together with furin, is the convertase for integrins α4, α5, α6 and αv (Bergeron et al., 2003; Lissitzky et al., 2000; Stawowy et al., 2004), suggesting that it may be important for cell adhesion. Furin has been implicated in the processing of a number of precursor proteins mediating the constitutive secretory pathway. Prototypical furin substrates include nerve growth factor (NGF) (Bresnahan et al., 1990), transforming growth factor-β (TGF-β) (Dubois et al., 2001; Dubois et al., 1995), insulin-like growth factor I (IGF-1) (Duguay et al., 1995) and IGF-1 receptor (Khatib et al., 2001; Lehmann et al., 1998). Furin is reportedly also involved in the processing-activation of proproteins from pathogens such as the envelope glycoprotein gp160 of the human immunodeficiency virus (HIV) (Decroly et al., 1996; Hallenberger et al., 1992; Moulard and Decroly, 2000), influenza virus haemaglutinin (Stieneke-Grober et al., 1992), cytomegalovirus glycoprotein B (Vey et al., 1995), Pseudomonas exotoxin A (Gu et al., 1996) anthrax toxin protective antigen (Molloy et al., 1992) and *Aeromonas hydrophila* aerolysin (Abrami et al., 1998). Other PCs negotiating the constitutive secretory pathway such as PC7 and PACE4 may participate in these proteolytic events.

TABLE 3. *Functional Classification of Proprotein Convertase Substrates*

PRECURSORS TO:	REPRESENTATIVE PRODUCTS
Hormones	α–MSH, β–LPH, ACTH, Gastrin, Glucagons, GLP-1, insulin, MIS, PTH, PP
Neuropeptides	7B2, Dynorphin, Enkephalin, β–End, Galanin, GnRH-I/II, hypocretins, Nociceptin/Orphanin, MCH, NPY, NPYY, PACAP, PACAP-RP, PRL-RP, SAAS, Somatostatin, SP, TRH
	BDNF, EGF, IGF-1/2, Lefty, NGF, NT3, NT4/5, PDGF-A/B, TGFβ
Enzymes	ADAM-10 (Kuz) ADAM-17 (TACE), renin, stremolysin-3
Receptors	IGF-I receptor, Insulin receptor, integrins α3/4/5/6//7/IIb, PTPm, LRP
ECM proteins	Cadherin-15, collagens α1(V)/VI, collagen 2(XI), Ng-CAM
Plasma proteins	Albumin, Protein C
Coagulation proteins	vWF, Factors IX/X

TABLE 3. *Functional Classification of Proprotein Convertase Substrates (Continued)*

PRECURSORS TO:	REPRESENTATIVE PRODUCTS
Transcription factors	Notch-1-receptor
Viral coat glycoproteins	HIV-1 gp 160, Influenza virus HA, CMV gb, VZV gp, CMV gp, Mumps Virus, YFV F protein, Ebola virus gp
Bacterial toxins	B. anthracis PA, Diphteria, Aerolysin
Others	Seminal Vesical Protein-1, Protein 3, ZP, SRM-1, Sortilin, Fibrillin

Abbreviations: ADAM, a disintegrin and metalloproteinase-like; B. anthracis PA, Bacillus anthracis protective antigen; BDNF, brain derived neurotrophic factor; CAM, cell adhesion molecule; CMVgb, cytomegalovirus glycoprotein b; GLP-1, HA, hemagglutinin; LRP, low-density-lipoprotein receptor-related protein; glucagon-like peptide; MCH, melanocyte-concentrating hormone; MIS, Mullerian inhibiting substance; NPY, neuropeptide Y; NPYY, neuropeptide Y receptor; NT3, neurotrophin-3; NT4/5 neurotrophin 4/5; PP, pancreatic polypeptide; PRL-RP, prolactin receptor precursor; PTH, parathyroid hormone; PTP, phosphotyrosine phosphatase; SP, substance P; TRH, thyrotropin releasing hormone; VZV gp, Varicella Zoster virus glycoprotein; YFV, yellow fever virus; ZP, zona pellucida

1.10 Biological Relevance of Proprotein Convertases

From the variety of substrates relying on PCs for their activation, it can be inferred that these enzymes are critical for nearly all biological processes. Their cellular multiplicity may be an evolutionary mechanism to insure redundancy of these most important physiological mediators. The importance of PCs in health and disease have been proposed shortly after their discovery (Chretien et al., 1995; Mbikay et al., 1993). Ex vivo studies have shown that PCs are important mediators of cell proliferation and differentiation (Bassi et al., 2003; Croissandeau et al., 2002; Kayo et al., 1996; Khatib et al., 2001; Konda et al., 1997). However, it was the characterization of human and mouse genetic mutants lacking one specific PC gene that provided the strongest argument for biological relevance of PCs.

1.10.1 PC1 Deficiency in Humans

Two patient models of PC1 deficiency have been reported. The first case (Jackson et al., 1997) involves a Caucasian female who displayed massive childhood obesity, hypogonadotropic hypogonadism, postprandial hypoglycemia, hypocortisolism, gross abnormalities of the small-intestinal absorptive function and evidence of impaired processing of adrenocorticotropic hormone (ACTH) and insulin precursors. Genetic analysis revealed that she was a compound heterozygote for PC1 mutations at Gly593Arg which resulted in the retention of the inactive propeptide in the ER and A->C^{+4} in the donor splice site of intron 5 which caused exon skipping and a frameshift to a premature stop codon in the catalytic domain. The altered glucose homeostasis observed in the patient is likely due to the unprocessed insulin which displays reduced activity and a longer half-life. Likewise, impaired processing of POMC to ACTH may

account for the observed hypocortisolism. The hypogonadotropic hypogonadism may be linked to the impaired processing of gonadotropin-releasing hormone (GnRH).

The second reported case of PC1 deficiency was of a female infant whose phenotype was dominated by severe small-intestinal dysfunction, gross obesity and hypocortisolism (Jackson et al., 2003). Sequencing of PC1 revealed that the patient was also a compound heterozygote for Glu250stop and Ala213del. The former mutation predicted to truncate the PC1 protein within the catalytic domain and the latter mutation resulting in the deletion of a highly conserved alanine within the same domain. PC1 deficiency is believed to cause intestinal malfunction through failure of maturation of propeptides within the endocrine cells and nerves throughout the gut.

1.10.2 Mouse Knockout Models

Mouse models of heritable PC deficiency have been generated for all PCs but PC5. Besides establishing the developmental relevance of PCs, they have provided physiological links between PC specific precursor proteins. The phenotypes of these mouse models have been recently reviewed (Taylor et al., 2003) (Table 4).

Furin-null mice die between embryonic days (e) e10.5 and e11.5. Their phenotypes include ventral closure defects, failure to undergo axial rotation, abnormal yolk sac vasculature and failure of chorioallantoic fusion (Constam and Robertson, 2000b; Roebroek et al., 1998). PACE4 gene ablation leads to a non-penetrant lethality phenotype with 25% of the embryos perishing between e13.5 and e15.5. Animals experience variable phenotypes such as heart, situs and craniofacial defects (Constam and Robertson, 2000a). In both furin and PACE4 null mice, the precursor to Nodal, a

TGF-β-related morphogen expressed in the epiblast and visceral endoderm of developing embryos, remain unprocessed (Constam and Robertson, 2000b).

PC7-nulls show no abnormal phenotype (Mbikay, M. personal communication). PC4-null male mice are infertile while females display subfertility (Mbikay et al., 1997). Male infertility may be due to sperm premature acrosome and its inability of sperm to attach to eggs (Gyamera-Acheampong et al., 2004). The subfertility of female is associated with impaired folliculogenesis. PC4-null testes and ovaries contain no processed forms of pituitary adenylating cyclase-activating protein (PACAP) (Li et al., 2000), indicating that this convertase is the sole pro-PACAP convertase in the gonads.

PC2-null mice, although viable, exhibit delayed growth (Furuta et al., 1997). The impaired processing of precursors to, among others, insulin (Furuta et al., 1998), glucagon (Furuta et al., 2001), somatostatin (Winsky-Sommerer et al., 2003), neurotensin (Villeneuve et al., 2002), αMSH (Laurent et al., 2004), and cholecystokinin (Rehfeld et al., 2002; Vishnuvardhan et al., 2000). These multiple neuroendocrine defects suggest that these mice may show subtle neuroendocrinopathies if closely monitored. Interestingly, ablation of the gene for the PC2 chaperone 7B2 results in a more severe phenotype associated with ACTH hypersecretion and a Cushing-like disease (Sarac et al., 2002; Westphal et al., 1999), suggesting that 7B2 may play other PC2-unrelated functions.

PC1 gene ablation also causes a non-penetrant phenotype of pre and perinatal lethality; associated with dwarfism and chronic mild diarrhea in survivors (Zhu et al., 2002a; Zhu et al., 2002b). The mutation results in impaired production of, among others, growth hormone-releasing hormone (GHRH) (Zhu et al., 2002b), insulin (Zhu et al.,

2002a), glucagon-like peptides (GLP) 1 (Ugleholdt et al., 2004) and cholecystokinin (CCK) (Cain et al., 2004). Interestingly, a 30-kb deletion in the PC1 gene was found to be homozygous lethal. The mutation was transmitted in a non-mendelian fashion among the offspring of heterozygote intercrosses, yielding no homozygous mutants, 80-90% heterozygous mutants and 10-20 % homozygous wild type. No PC1-nulls were identified among blastocysts of these intercrosses, suggesting that they died during preimplantation development (Dr. Mbikay, unpublished data). This observation led us to examine the expression of PC1 during mouse preimplantation embryonic development.

1.11 PC Relevance during Preimplantation Embryogenesis

1.11.1 Embryogenesis

Embryogenesis is the process of embryo formation from undifferentiated cells. Embryo formation is initiated from the fertilization of a mature egg with a sperm. The embryo then proceeds to develop up to the stage of implantation without increasing in mass. Preimplantation development include cleavage of the fertilized ovum, compaction (morula formation), and cavitation (blastocyst formation) (Figure 5).

Described below are the major events of mouse embryonic development (Hogan, 1994). At a peak of luteinizing hormone (LH), selected ovarian oocytes undergo nuclear maturation and their first meiotic division resulting in the extrusion of the first polar body and release from the follicle.

TABLE 4. *Phenotypic and Molecular Defects in Proprotein Convertase-Deficient Mice*

CONVERTASE	NULL PHENOTYPE	MOLECULAR DEFECTS
Furin	Embryonic lethality at e10.5-11.5, ventral closure defects, failure to undergo axial rotation, abnormal yolk sac vasculature, failure of chorioallantoic	Perturbed expression of *pitx2* and *lefty2*
PACE4	Non penetrant embryonic lethality at e13.5-15.5, heart situs and craniofacial defects	Abnormal expression of axis determining genes *nodal* and *lefty*
PC1 (model a)*	Pre-and postnatal lethality, dwarfism, chronic mild diarrhea	Decreased growth hormone (GH) mRNA and circulating GH, increased POMC mRNA and impaired processing of proglucagon processing and proinsulin
PC1 (model b)*	Preimplantation embryonic lethality	
PC2	Retarded post partum growth, hypoglycemia	Multiple defects of neuroendocrine proprotein processing
PC4	Reduced fertility	Impaired proPACAP processing
PC5	Embryonic death at e10.5-11.5	Not available
PC7	No abnormal phenotype	None reported

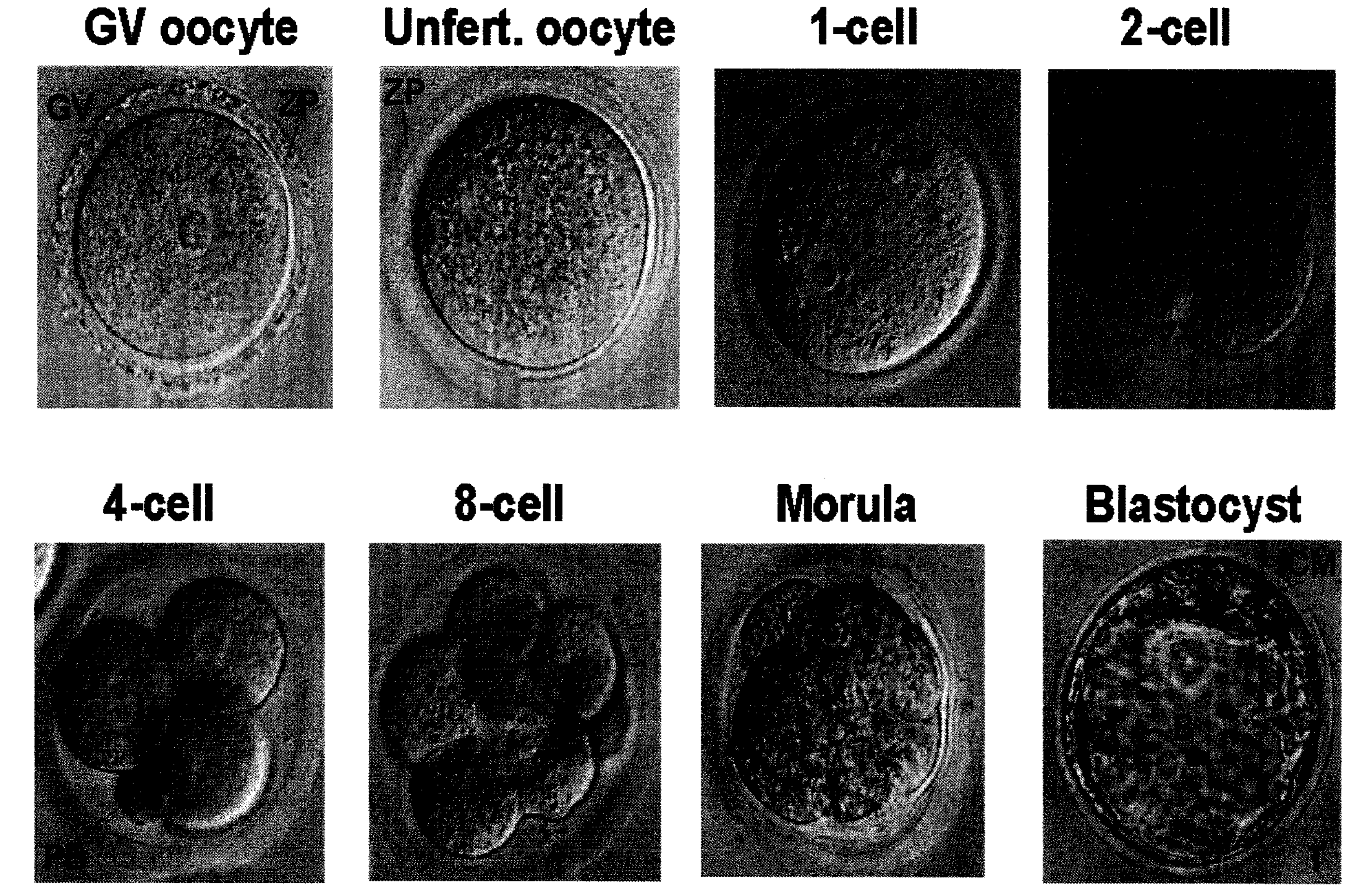
*Model a: <1 kb deletion. Model b: 30 kb deletion.

Fertilization of an egg by sperm results in a second meiotic division, followed by the formation of nuclear membranes around both maternal and paternal chromosomal material. The newly fertilized embryo contains two pronuclei, one male and one female, each housing a haploid set of chromosomes from each parent. These pronuclei migrate toward each other to the center of the egg where their membranes break down and reorganization of the cytoskeleton occurs in preparation for the first cell cleavage. Maternal transcripts are responsible for the first cleavage of the embryo to the 2-cell stage, while beyond this stage maternal mRNA becomes degraded and replaced by the embryonic genome. Cleavages proceed to produce 4-cell, 6-8 cell, morula and finally blastocyst embryos.

Blastocyst formation marks the appearance of the first two tissue lineages, the trophectoderm (TE) and primitive ectoderm. The blastocyst sheds its outer coat, the zona pellucida (ZP), exposing the TE cells which attach to the uterus lining and initiate implantation.

After implantation, the epiblast cells of the primitive ectoderm dramatically increase the rate of growth of the embryo. Approximately 5 to 10 days post fertilization, three primary germ layers emerge known as the ectoderm, mesoderm and definitive endoderm are produced during gastrulation. These layers establish the basic body plan and organ primordia of the mouse. Development continues with the notochord moving to the midline of the anteroposterior axis and the paraxial mesoderm on either side dividing up into somite blocks.

FIGURE 5. *Preimplantation Embryo Development* All stages of mouse preimplantation development. GV-germinal vesicle, ZP-zona pellucida, PN-pronuclei, PB-polar body, ICM-inner cell mass, B-blastocoel cavity, T-trophectoderm. Courtesy of Dr. Jay Baltz's lab.


GV oocyte
GV
ZP
Unfert. oocyte
ZP
1-cell
2-cell
4-cell
PB
8-cell
Morula
Blastocyst
ICM
T

The neural plate folds upward into the neural tube consisting of forebrain, midbrain, hindbrain and spinal cord. From the ectoderm surface stems the nose, ears and lens while the neural crest cells migrate and the heart, circulatory system and limb buds form. During this 19-20 day gestation period, the genes which code for differentiation and morphogenesis are active.

1.11.2 PC Substrates in Preimplantation Embryos

Preimplantation embryo development is known to be influenced by autocrine factors (Diaz-Cueto and Gerton, 2001; O'Neill, 1997). These embryos express several such factors known to be generated through PC activation of their cognate precursors. These factors include neuropeptides, cytokines, growth factors and their receptors as well as integrins.

Neuropeptides --- GnRH and its receptor are expressed in preimplantation embryos from the morula to hatching blastocyst stages (Casan et al., 1999; Raga et al., 1999; Raga et al., 1998). Preimplantation embryonic development in culture was shown to be stimulated by exogenous GnRH and inhibited by a GnRH antagonist (Raga et al., 1999). Furthermore antisense inhibition of the translation of mRNA for GnRH has been shown, *in vitro,* to block development of these embryos (Raga et al., 1999). Ocytocin and its receptor are also expressed preimplantation (Stock and Osterlund, 1998). ProGnRH and pro-oxytocin/vasopressin are potential substrates for convertases (Coates and Birch, 1998; Rangaraju and Harris, 1993; Wetsel et al., 1995).

Cytokines --- Cytokines of the TGF-β family are produced by mouse preimplanatation embryos. Transcripts of TGF-β1 and 2 are detectable in embryos after

activation of the zygotic genome (Paria et al., 1992; Rappolee et al., 1988). Transcripts for signalling type I and type II receptors for TGF-beta are detectable and were shown to be present in fertilized oocytes and at the blastocyst stage (Roelen et al., 1998). It has been shown using radiolabeling techniques that embryos can specifically bind TGF-β1 and 2 from the 8-cell to the blastocyst stage. Concordantly, the addition of TGF-β into culture media has been shown to increase embryonic growth suggesting that it may play a role in the regulation of differentiation (Rappolee et al., 1988).

Growth factors -- Insulin and IGF-I receptors are expressed at the 8-cell stage of mouse preimplantation development (Murphy and Barron, 1993). IGF-II transcripts and its receptor have been detected in 2-cell mouse embryos and its protein in the blastocyst embryo (Harvey and Kaye, 1991; Rappolee et al., 1988). Insulin, IGF-I and IGF-II have been shown to stimulate cell proliferation and blastocyst formation in cultured mouse embryos (Murphy and Barron, 1993; Watson et al., 1992). They also mediate protein synthesis at the preimplantation level (Harvey and Kaye, 1988; Pantaleon and Kaye, 1996). Platelet derived growth factor-A (PDGF-A) and α-receptor are present in mouse preimplantation embryos from the 2-cell to the blastocyst stage (Palmieri et al., 1992; Watson et al., 1992). It has been reported that PDGF is able to increase trophoblast outgrowth from mouse blastocysts when supplemented to the culture media (Haimovici and Anderson, 1993).

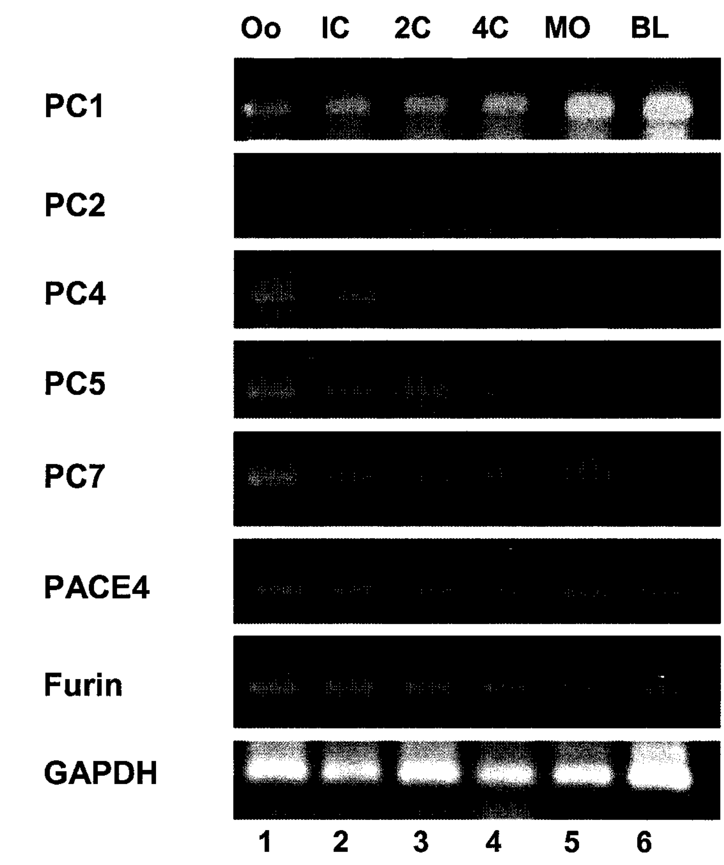
Integrins --- Several integrins are expressed in preimplantation embryos, among them integrins αv, α4, β5, α6, β1, β3 and β4 (Bloor et al., 2002; Campbell et al., 1995; Dubey et al., 2001). Integrins reportedly play important roles in blastocyst implantation

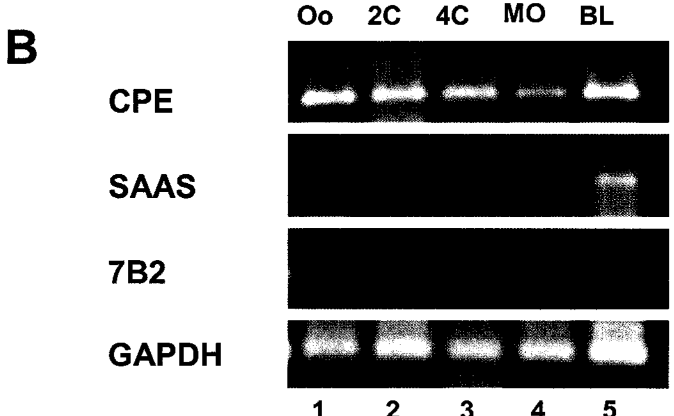
(Kimber and Spanswick, 2000; Wang et al., 2002). Alpha integrins are activated by PCs, an indication that these enzymes contribute to this process.

1.11.3 Proprotein Convertases in Embryogenesis

Our laboratory has used reverse transcription-polymerase chain reaction (RT-PCR) (experimentation conducted by Gilles Croissandeau) to examine the expression of all seven PCs and their companion proteins CPE, 7B2 and SAAS in eggs and preimplantation embryos. As shown in Figure 6A, transcripts for all the PCs, except PC2 were detected at some point in eggs and at some point in developing embryos. Although the RT-PCR was not designed to be quantitative, the electropherogram and PC/GAPDH amplicon intensity ratios (GAPDH mRNA serving as an internal control), clearly indicate distinct patterns of these transcripts. Transcripts for PC4 and PC5 were detected only in eggs and early conceptuses (fertilized oocyte, 1-cell and 2-cell embryos). Transcripts for PC1, PC7, PACE4 and furin were detected at all stages. Interestingly, PC1 transcripts appeared to be the most abundant and to increase from the oocyte to the blastocyst stage (Figure 6A), suggesting that this enzyme may be particularly important during preimplantation development. CPE transcripts were present in comparable amounts at all stages; 7B2 transcripts were undetectable, SAAS transcripts were readily detectable in blastocysts only (Figure 6B).

FIGURE 6. *Transcripts of PCs and their Companion Proteins in Preimplantation embryos.* Eggs and embryos were pooled by developmental stage (about 100 for eggs and 1-cell embryos, 50 for 2-cell, 4-cell, morula and blastocyst stage embryos. Total RNA was extracted using the Trizol reagent as recommended by the manufacturer (Life Technologies, Burlington, Ontario). The RNA was treated with RNase-free DNases I (Life Technologies) and 2 μg were reverse-transcribed into cDNA using the SuperScript II Reverse Transcriptase (Life Technologies). Three microliters of the reverse transcription reaction were utilized for PCR amplification of cDNA fragments for all PCs, CPE, SAAS, 7B2 or glyceraldehyde phosphate dehydrogenase (GAPDH). The sequences of the PCR primers are shown in Appendix 1. The PCR reaction mixes contained 0.5 units of rTaq DNA polymerase (Life Technologies), 1X PCR buffer, 0.2 mM dNTP, 0.5 μM of sense and antisense primer for a specific cDNA in a 50-μl volume. PCR was performed for a total of 40 cycles. Each cycle involved a 94°C/1 min polymerization step. After a 5-min incubation at 72°C, the reaction was quenched at 4°C. The PCR products were electrophoresed on agarose gels, stained with ethidium bromide and revealed by UV irradiation. The authenticity of the amplified sequences was verified by restriction enzyme mapping. Oo, 1C, 2C, 4C, MO and BL, stand respectively for oocyte, 1-cell embryo, 2-cell embryo, 4-cell embryo, morula and blastocyst. This experiment was repeated on three separate occasions with similar results.


A
Oo
IC
2C
4C
MO
BL
PC1
PC2
PC4
PC5
PC7
PACE4
Furin
GAPDH
1
2
3
4
5
6


B
Oo
2C
4C
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CPE
SAAS
7B2
GAPDH
1
2
3
4
5

Taken together, these results indicate that various components of the prohormone/proneuropeptide conversion system are present and potentially functional in preimplantation embryos.

To our knowledge, expression of PCs during mammalian preimplantation embryo development has never been examined at the protein level.

1.12 Statement of Objectives

The following study was initiated after transcripts for PCs and their companion enzymes had been detected in our laboratory by RT-PCR in mouse preimplantation embryos. The relative abundance of PC1 mRNA, together with the observation of preimplantation embryonic lethality of mice carrying a large PC1 gene deletion, suggested to us that PC1 is important during mouse preimplantation embryonic development. Further investigation of expression of this convertase at this stage was therefore warranted. Our objectives were:

1. **to determine the molecular form of PC1, CPE and SAAS in mouse eggs and preimplantation embryos;**
2. **to establish the subcellular localization of these molecules at these developmental-stages;**
3. **having observed that PC1 can transiently translocate to pronuclei, to identify the nuclear localization signal (NLS) responsible for this translocation.**

2.0 MATERIALS AND METHODS

2.1 Animals and Materials

Seven to eight-week old CF1 mice were obtained from Charles River (Wilmington, MA). They were housed in a pathogen-free facility on a 12-h light/dark cycle with free access to food and water. Experimental manipulations on these mice were approved by the Animal Care Committee of the Ottawa Health Research Institute and performed in accordance with the guidelines of the Canadian Council on Animal Care. The antibodies used in this study are described in Appendix 2.

2.2 Oocyte and Embryo Collection, Embryo Culture

Female mice were induced to superovulate by intraperitoneal injection of 5 international units (IU) of pregnant mare serum gonadotropin (PMSG) followed, 48 h later, by 5 IU human chorionic gonadotropin (hCG). They were individually caged with male mice to mate overnight. The next day, those showing a vaginal plug were sacrificed by cervical dislocation. The oviducts were surgically removed and transferred into a drop of Hepes/KSOM/PVA/hyaluronidase medium [KSOM medium (95 mM NaCl, 2.5 mM KCl, 0.35 mM KH_2PO_4, 0.2 mM $MgSO_4.7H_2O$, 10 mM Na lactate, 0.2 mM glucose, 0.2 mM Na-pyruvate, 4 mM $NaHCO_3$, 1.7 mM $CaCl_2.2H_2O$, 1 mM glutamine, 0.01 mM EDTA, 240 mOsM, 0.16 mM K-penicillin G, 0.03 mM streptomycin sulfate) containing 21 mM Hepes, 0.01% polyvinyl alcohol (PVA) and 1 mg/ml hyaluronidase]. The oviducts were torn at the swollen ampulla with forceps and a 26-gauge sterile needle; fertilized eggs were removed and transferred from the

hyaluronidase-containing drops to fresh Hepes-KSOM/PVA drops using a mouth-operated pipet. They were washed by successive passages through 4 drops of the same buffer and then transferred into a separate culture dish containing 3 drops of KSOM/PVA under mineral oil (Lawitts and Biggers, 1993). They were transferred from one drop to the next for washing and left in the final drop to incubate at 37°C under a 5% CO_2/95% air atmosphere until they reached the blastocyst stage. Alternatively, unfertilized eggs were collected from females that were superovulated but not mated 24 h post hCG; 1-cell, 2-cell, 6/8-cell, morula and blastocysts were collected from superovulated and mated females, 28-, 32-, 72-, 98- and 120 h post hCG, respectively.

2.3 Immunoblotting

Equal numbers of eggs or stage-specific embryos were pooled into an equal volume of gel loading buffer (0.125 M Tris-Cl, 4% SDS, 20% v/v glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8) and boiled. Proteins were fractionated by electrophoresis through an 8 % polyacrylamide/Tris-glycine gel. They were electrotransferred onto a nitrocellulose membrane at 60 V for 3 h. The membranes were rinsed for 10 min in PBS containing 0.05% Tween-20 (PBST). They were incubated at room temperature for 1 h in PBST containing 5% skimmed milk (blocking buffer) to block non-specific binding sites, then for 60 min in blocking buffer containing a rabbit or mouse primary antibody and, after five 5-min rinses in PBST, for 1 h in blocking buffer containing horseradish peroxidase (HRP)-conjugated secondary antibody against either rabbit or mouse IgGs. They were washed 5 times for 5 min in PBST and then

probed for HRP-conjugates using the Western Lightning Chemiluminescence Reagent Plus Kit as specified by the manufacturer (Perkin-Elmer, Boston, MA).

2.4 *Immunofluorescence Laser Confocal Microscopy*

Eggs and embryos were incubated for 20 min at 37°C in 0.5 ml of a pre-warmed fixation solution [2% formaldehyde, 0.5% Triton X-100 in TBS (10 mM Tris pH 7.5, NaCl 150 mM)]. After three 15-min rinses in 0.5 ml of blocking solution (TBS containing 2% BSA, 2% FBS, 0.1 M glycine, 0.01% Triton X-100), they were incubated overnight with a rabbit or mouse primary antibody, rinsed four times for 15 min in the blocking buffer and incubated for 1 h in the dark with an Alexa Fluor^{594} or Alexa-Fluor^{488}–conjugated goat IgG against rabbit or mouse IgGs, respectively. After five 15-min rinses with 500 μl of blocking buffer in the dark, they were dried on coverslips, imbedded in 50 % glycerol (v/v) in water and attached to slides. For control samples, treatment with a primary antibody was omitted. Some embryos were counterstained overnight at room temperature with 2 mM SYTOX Green (Molecular Probes, Eugene, OR) in 0.5 X TBS-0.25 % Triton X-100. Confocal images were collected using an Olympus 1 X70 inverted microscope equipped with a BioRad MRC-1024 confocal laser-scanning unit. For the Alexa^{594}-stained samples, excitation at 594 nm was produced with a helium-neon laser and emission was captured at 600 nm EFLP. For Alexa^{488} or SYTOX Green-stained samples, excitation at 488 nm was produced with an argon ion laser and emission was captured through a band pass filter centered at 522 nm. Images were collected using an Olympus UApo 40X (NA 1.15) water immersion lens. Sections were taken every 0.5 μm (Z-series) through the thickness of the egg or embryo.

Transmitted light was also collected to obtain bright field images of each egg or embryo analyzed.

2.5 *Immunohistochemistry*

Cultured cells (HEK293 and LoVo) were grown in 1-cm^2 slide chambers. After removal of culture medium, attached cells were rinsed once in PBS, fixed with 4% paraformaldehyde in PBS for 15 min, washed three times for 10 min with PBS in a glass chamber with stirring, permeabilized with 0.5% Triton-X-100 in PBS for 15 min, washed with PBS as above, incubated for 1 h with a primary antibody in 3% BSA in PBS, washed as above but in PBS containing 0.1% Tween-20, incubated with $Alexa^{593}$-conjugated antibody in the PBST/Tween buffer for 1 h in the dark, washed with this buffer in the dark and finally rinsed with water. To stain for nucleic acids, a drop of mounting medium containing 1.5 μg/ml of 4', 6-diamidino-2-phenylindole (DAPI, Vectashield, Burlingame, CA) was applied to a coverslip and the latter was attached to the slide. Fluorescence was monitored using a Carl-Zeiss microscope coupled to an Axioplan 2 imaging system. Images were collected using a Zeiss Plan-NEOFLUAR 40X and 63X 1.30 oil immersion lenses.

2.6 *Construction and Transfection of a Prepro^{PC1}-EGFP Expression Vector*

The DNA sequence encoding the prepro domain was amplified from a human PC1 cDNA plasmid (forward primer, 5'-TCG AAT TCT GTG AGC TAT GGA GCG AAG A-3'; reverse primer, 5'-GTG GAT CCG AGT CCC TTA GAG CTG AAC GT-3'). The primers introduced a *Bam*H1 and an *Eco*R1 sites at the 5' and 3' ends of the amplicon (underlined), respectively. The amplicon was digested with these two enzymes to generate cohesive ends. It was cloned into the pEGFP-N1 vector (Clontech, Mississauga, ON) cleaved with the same enzymes within the multiple cloning site. The resulting plasmid (pPrepro^{PC1}-EGFP) contained the cDNA for the preprodomain of PC1 in an open reading with the gene for Enhanced Green Fluorescent Protein (EGFP), under the human cytomegalovirus promoter-enhancer. Other plasmid constructs used in this study include pCMV-PC1^{WT} and pCMV-PC1^{G493R}, for transduction of wild type PC1 and an inactive mutant PC1 carrying a G483R mutation in its P domain (Jackson et al., 2003; Jackson et al., 1997), respectively.

2.7 *Transient Transfection*

Two days prior to transfection, human embryonic kidney HEK293 cells and human colon carcinoma LoVo cells were plated in 10-cm Petri dishes at ~5x10^{6} cells/dish in 8 ml of HAM F12 medium containing 10% fetal bovine serum (FBS). Fresh FBS-free medium was substituted. For each dish, two separate mixtures of transfection reagents were prepared: the first contained 1.5 μg of plasmid vector in 800 μl of Opti-MEM medium (GIBCO Invitrogen Coorporation, Grand Island, NY); the second was

prepared by mixing 100 μl Lipofectamine Reagent (Life Technologies) with 800 μl of Opti-MEM. The two mixtures were combined and layered over cells. After 2 h of incubation, 8 ml of HAM F12/10% FBS were added to each dish and incubation was resumed for 48 h.

2.8 Subcellular Fractionation

Each monolayer of transfected cells was rinsed twice with 1 ml of cold PBS-D (0.14 M NaCl, 0.013 M $Na_2HPO_4 \cdot 7H_2O$, 2 mM $NaH_2PO_4 \cdot H_2O$ in H_2O). Cells were scraped off the plate into 1 ml of a homogenization buffer (HB, 10 mM Tris-HCl pH 8.0, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.15% Triton X-100, 0.5 mM DTT) containing 0.3 M sucrose. They were transferred into a Down homogenizer on ice and homogenized with 20 pestle strokes. Half of the homogenate (0.5 ml) was layered over a 0.4 ml cushion of 0.4 M sucrose in HB in an Eppendorf tube. After centrifugation at 660 *g* for 10 min. at 4°C, the upper cytoplasmic fraction was transferred into a clean tube; the sucrose cushion was discarded; the pellet of nuclei was suspended in 0.5 ml of PBS containing 1X Protease Inhibitor Cocktail (PIC, Roche, Mannheim, Germany) and sedimented again as above. The initial homogenate (0.5 ml) and the cytoplasmic fraction (0.5 ml) were each mixed with 50 μl of 100% (w/v) trichloroacetic acid (TCA) and incubated on ice for 1 h to allow proteins to precipitate. Precipitates were pelleted by centrifugation for 5 min at 20,800 *g* at 4°C. Pellets were washed twice in 0.5 ml of ice-cold acetone and air-dried.

Nuclear and cytoplasmic proteins were dissolved in 20 μl of Celis Buffer (9.8 M urea, 2% Nonidet P-20, 1% Triton X-100 and 1X PIC). After a 1-min sonication,

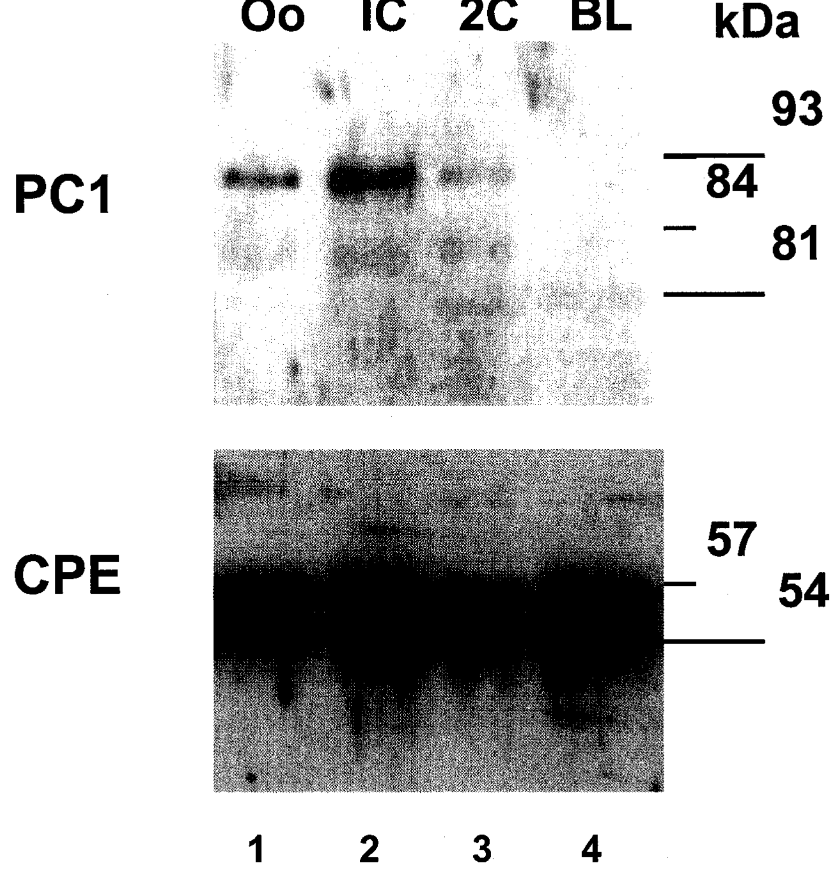
insolubles were removed by centrifugation at 20,800 *g* for 5 min at 4°C. Supernatants were collected and analyzed by immunoblotting.

3.0 RESULTS

3.1 Molecular Forms of PC1 and CPE

To determine which molecular forms of PC1 are found in eggs and early embryos, we conducted immunoblot analysis of unfertilized eggs, 1-cell and 2-cell embryos as well as of blastocysts, using an antibody against the C-terminal region of PC1 (see Figure 4). The antibody recognized PC1 forms of 93, 84 and 81 kDa in unfertilized eggs, in 1-cell and 2-cell embryos. (Figure 7, upper panel, lanes 1, 2 and 3). Blastocysts contained barely detectable levels of the 81-kDa form (Figure 7, upper, lane 4), probably because most of the larger form had been converted to the 75-kDa and the 66-kDa forms by C-terminal cleavages (see Figure 4). This possibility could not be verified because the antibody against the N-terminal region of mature PC1 was not sensitive enough for immunoblotting. An immunoblot for CPE revealed the typical forms of 54 and 57 kDa, at comparable levels in all the samples (Figure 7, lower panel). The presence of both PC1 and CPE suggests that their substrates can be fully processed during early development. The molecular forms of SAAS could not be verified for lack of a suitable antibody.

FIGURE 7. *Molecular Forms of PC1 and CPE.* Specific immunoblotting was conducted using an antibody against the C-terminal region of PC1 (upper panel) and of CPE (lower panel). The same number of eggs or embryos (100) was used in each lane. 1C, 2C and BL, stands respectively for 1-cell embryo, 2-cell embryo and blastocysts. The 93 kDa form of PC1 is detected at the Oo, 1C and 2C stage, the 84 kDa form of PC1 is detected at the Oo, 1C and 2C stage, and the 81 kDa form of PC1 is detected at the 2C and BL stage. This experiment was repeated three times with similar results.


Oo
IC
2C
BL
kDa
93
PC1
84
81
CPE
57
54
1
2
3
4

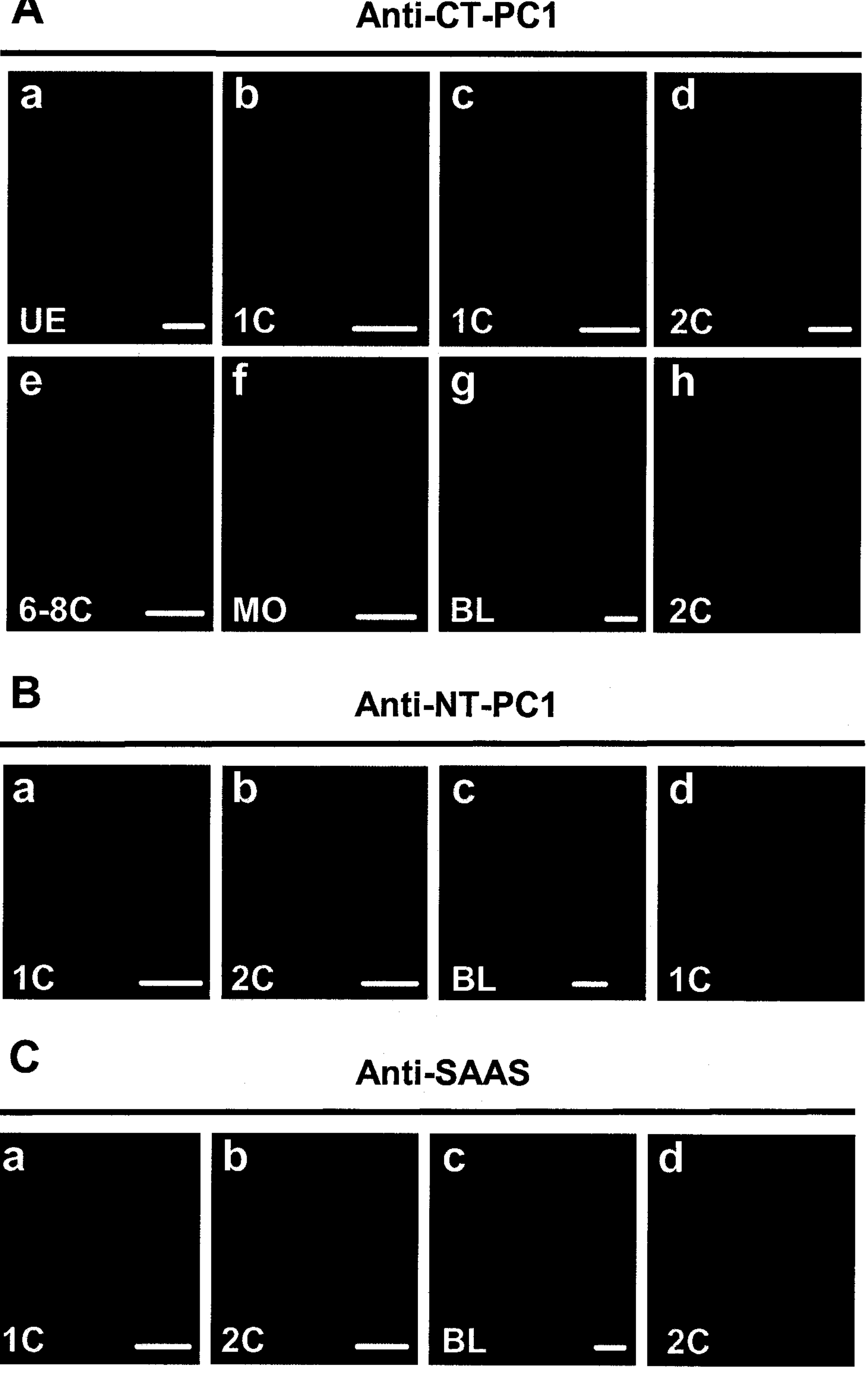
3.2 *Subcellular Localization of PC1 and SAAS*

We used immunofluorescence laser confocal microscopy to examine the distribution of PC1 in eggs and embryos. The antibody against the C-terminal region of PC1 evenly stained the cytoplasm of unfertilized eggs (Figure 8A, panel a). Most surprisingly, in 28-h post-hCG fertilized eggs, the staining was primarily localized in pronuclei (Figure 8A, panels b and c). In 2-cell embryos, the staining was observed throughout the cytoplasm (Figure 8A, panel d). It was perinuclear and punctate in 6 to 8-cell embryos (Figure 8A, panel e) and was most intense at blastomere junctions in morula and blastocysts (Figure 8A, panels f and g).

The antibody against the N-terminal region of PC1 evenly stained pronuclear stage 1-cell embryos (Figure 8B, panel a), suggesting that only PC1 forms carrying an intact C-terminus were selectively translocated to pronuclei. In 2-cell embryos, the staining was unevenly distributed and relatively more intense in the cytoplasm (Figure 8B, panel b). In blastocysts, it appeared to be concentrated in the trophectoderm and the inner cell mass (Figure 8B, panel c).

SAAS staining pattern was similar to that obtained with the anti-NT-PC1 antibody at the three developmental stages examined (Figure 8C, panels a-c). Consistent with the levels of SAAS transcripts (see Figure 6), SAAS staining in 1-cell embryos were significantly weaker than in blastocysts.

FIGURE 8. *Subcellular Localization of PC1 and SAAS.* Immunofluorescence laser confocal microscopy for PC1 and SAAS. (**A**) PC1-staining with the antibody against the C-terminal region. Striking pronuclear localization is observed in 1C embryos (panel b and c). Peripheral cell staining in the cell to cell junctions is observed in the BL (panel g). (**B**) PC1 staining with an antibody against the N-terminal region. (**C**) ProSAAS staining. BL has analogous cytosolic and cell to cell junction staining (panel c) as that of C-terminal PC1 stained BL (**A**, panel g). Abbreviations for eggs and embryos are as indicated in Figure 6. Negative controls consisted of embryos processed in the same manner except for the fact that the primary antibody was omitted (**A**h, **B**d, **C**d). Bar = 15 μ.m. Localization patterns of PC1 and SAAS were determined from two repeated experiments for each stage of preimplantation development.

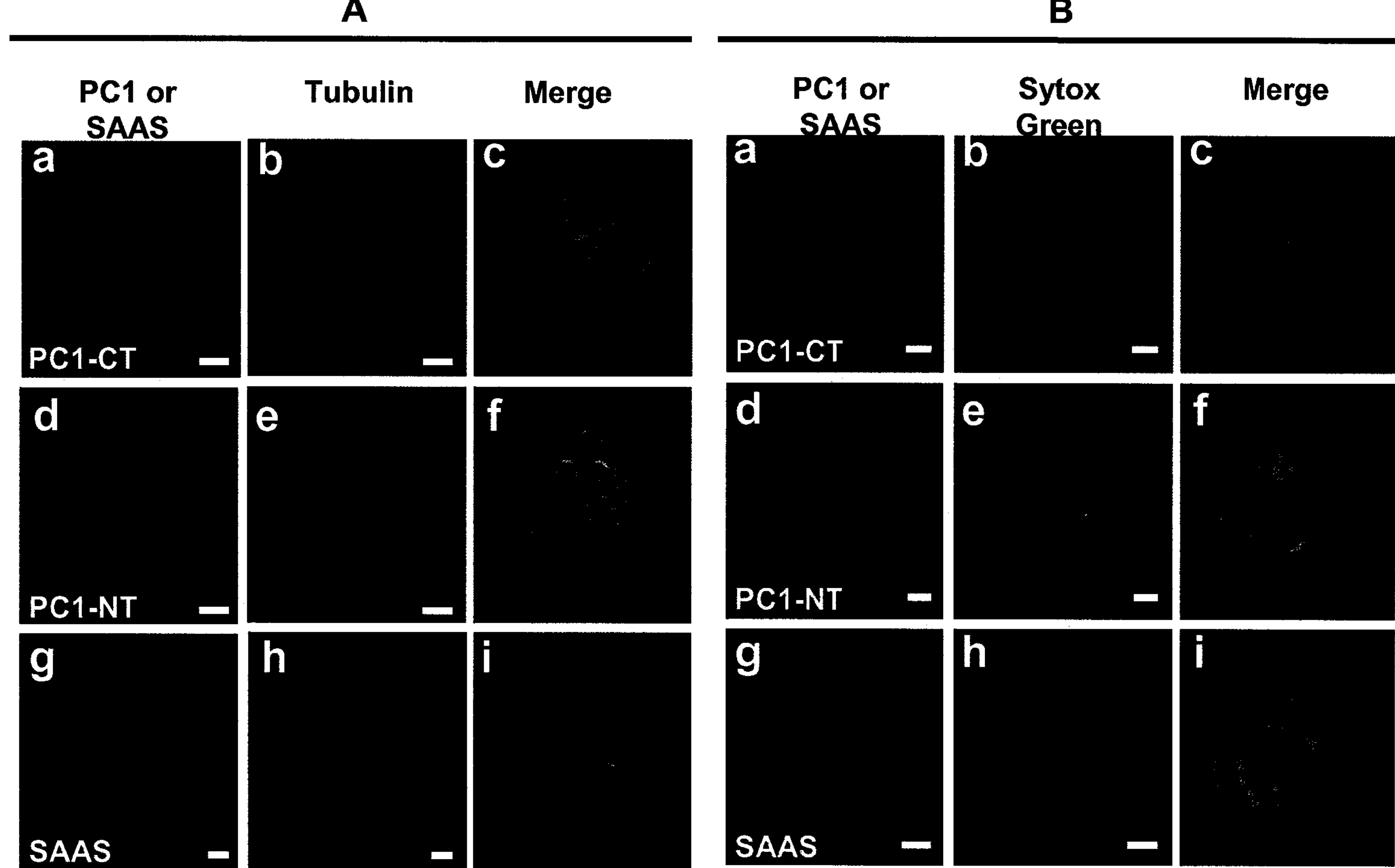

A
Anti-CT-PC1
a
UE
b
1C
c
1C
d
2C
e
6-8C
f
MO
g
BL
h
2C
B
Anti-NT-PC1
a
1C
b
2C
c
BL
d
1C
C
Anti-SAAS
a
1C
b
2C
c
BL
d
2C

To verify the cytoplasmic and non-nuclear localization of PC1 and SAAS in blastocysts, these embryos were stained for PC1 or SAAS with Alexa^{493}-conjugated secondary antibody and for cytoplasmic β-tubulin with Alexa^{488}-conjugated secondary antibody or for nuclear DNA with SYTOX Green. Figure 9 shows individual fluorescence stainings for PC1 using the antibody against PC1-CT (Figures 9A and 9B, panel a), PC1-NT (Figures 9A and 9B, panel d), SAAS (Figures 9A and 9B, panel g), β-tubulin (Figures 9A, panels b, e and h) and nuclear DNA (Figure 9B, panels b, e and h). Merging of PC1 or SAAS-specific and tubulin-specific immunofluorescences (Figure 9A, panels c, f and i) indicated partial co-localization of these molecules in the cytoplasm. CT-intact PC1 forms and SAAS were extensively co-localized with β-tubulin in the cytoplasm (Figure 9A, panels c and i). A substantial amount of non-cytoplasmic staining was observed with the NT-PC1 antibody which recognizes all PC1 molecular forms (Figure 9A, panel f). Merging of PC1 or SAAS-specific immunofluorescence and DNA-SYTOX fluorescence indicated that CT-intact PC1 forms and SAAS were generally non-nuclear or perinuclear (Figure 9B, panels c and i), consistent with their cytoplasmic localization. In contrast, when the NT-PC1 antibody was used, a number of PC1-rich blastomeres stained positive for nuclear PC1 (Figure 9B, panel f).

3.3 ProPC1 can Translocate to the Nucleus in LoVo Cells.

Based on the relative abundance of the 93-kDa proPC1 in early embryos, we speculated that the presence of the prodomain was necessary for translocation of the zymogen to the pronuclei of fertilized eggs.

FIGURE 9. *Subcellular Localization of PC1 with Tubulin and Sytox Green.* Immunofluorescence laser confocal microscopy for co-localization of PC1 or ProSAAS (red stain in the first column, the antibodies are specified in panels a, d, and g) with **(A)** the cytoplasmic marker β-tubulin or **(B)** nuclear DNA stained with Sytox Green (green color in the middle column) in mouse blastocysts. The yellow stain in the merged images of the last column identifies areas of co-localization. PC1-CT and SAAS immunostained blasts (**A**a and g) overlap with cytoplasmic marker tubulin (panel **A**c and i) and are absent in the nucleus (**B**c and i). PC1-NT immunostained blasts overlap with both cytoplasmic marker tubulin and nuclear marker Sytox Green (**A**f and **B**f). Experiment was repeated at twice. Bar = 15 μ.m.


A
PC1 or SAAS
Tubulin
Merge
a
b
c
PC1-CT
d
e
f
PC1-NT
g
h
i
SAAS
B
PC1 or SAAS
Sytox Green
Merge
a
b
c
PC1-CT
d
e
f
PC1-NT
g
h
i
SAAS

Cleavage within the prodomain or between this domain and the catalytic domain of PC1 is primarily an intramolecular event (Shennan et al., 1995), but the presence of typical furin recognition sites within and at the C-terminus of the prodomain (RRSRR or RSKR, see Figure 4) raises the possibility that furin might cleave proPC1 in trans. To determine whether in the absence of these cleavages, the zymogen could be directed to the nucleus, expression vectors for active $PC1^{WT}$ or inactive $PC1^{G483R}$ were transiently transfected into HEK293 cells that contain active furin or into LoVo cells that lack furin activity (Takahashi et al., 1993; Takahashi et al., 1995) and have little of any other PC activities (Seidah et al., 1994; Seidah et al., 1996).

Transfected cells were analyzed by indirect immunofluorescence using an antibody against the N-terminus of PC1. In HEK293 cells both PC^{WT} and $PC1^{G483R}$ were predominantly localized in the perinuclear region, presumably the ER and the Golgi (Figure 10A, panels a and b). In LoVo cells, in contrast, they were detected in both the cytoplasm and the nucleus (Figure 10A, panels c and d). The mutant form was mostly nuclear (Figure 10A, panel d).

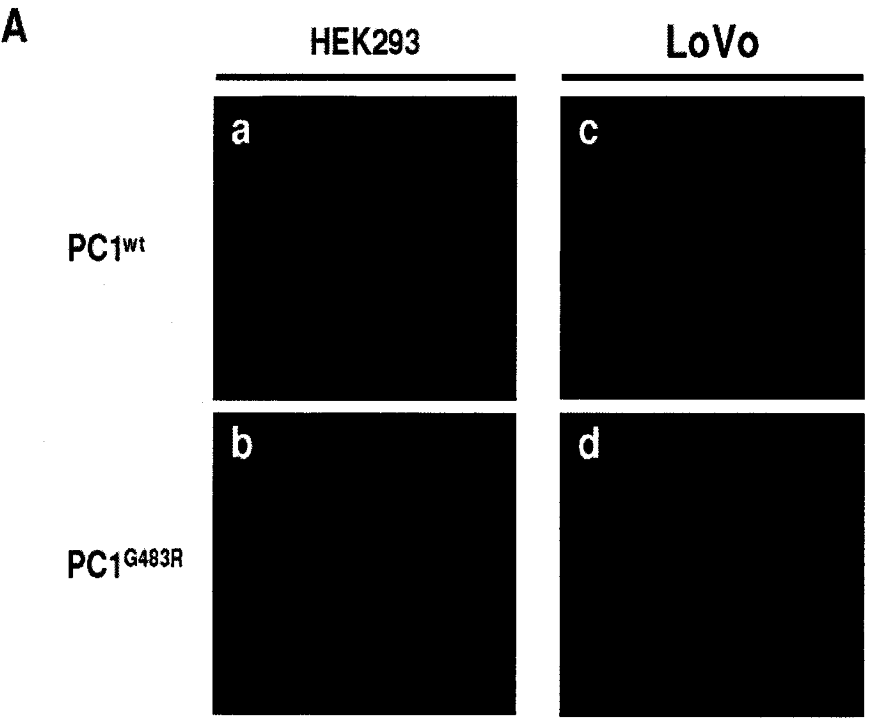
We also examined by immunoblotting the relative abundance of $PC1^{WT}$ and $PC1^{G483R}$ molecular forms in cytoplasmic and nuclear fractions of transfected LoVo cells. The 93-kDa $proPC1^{WT}$ was partially processed to the 85-kDa form whereas $proPC1^{G483R}$ remained unprocessed (Figure 10B, lanes 1, 3 and 5 vs 2, 4 and 6). All PC1 molecular forms were found in both the nuclear and the cytoplasmic fractions. However, most of the 84-kDa $PC1^{WT}$ was found in the cytoplasmic fraction (Figure 10B, lane 3 vs 5), whereas most of the 93-kDa form proPC1 was present in the nucleus fraction (Figure 10B, lanes 6 vs 4). These results, together with the

immunohistochemical observations, support the view that unprocessed proPC1 can translocate to the nucleus.

3.4 The prodomain of PC1 contains a putative nuclear translocation signal (NLS).

To determine whether the prodomain of PC1 was responsible for this nuclear translocation, we constructed a vector for expression of a fusion EGFP carrying the prepro sequence of human PC1 (Prepro^{PC1}, Figure 11A) at its N-terminus. This vector and the control empty vector were transiently transfected into LoVo cells. Total homogenates, cytoplasmic and nuclear subcellular fractions of the transfected cells were analyzed by immunoblotting for EGFP immunoreactivity. Unlike the 34-kDa EGFP, which was primarily found in the cytoplasmic fraction (Figure 11B, lanes 4 *vs* 6), most of 45-kDa pro^{PC1}-EGFP was contained in the nuclear fraction (Figure 11B, lanes 5 vs 3), indicating that the prodomain of PC1 has nuclear localization properties.

FIGURE 10. *ProPC1 can Translocate to the Nucleus in LoVo Cells.* (**A**) Immunofluorescence analysis for $PC1^{WT}$ (panels **a** and **c**) and $PC1^{G483R}$ (panels **b** and **d**) in transfected HEK293 cells (panels **a** and **b**) and LoVo cells (panels **c** and **d**), using an antibody against the N-terminus of PC1. Both $PC1^{WT}$ and $PC1^{G483R}$ are detected in the secretory pathway in HEK293 cells. $PC1^{G483R}$ is detected with a greater expression in the nucleus of LoVo cells than is $PC1^{WT}$. (**B**) Immunoblot of subcellular fractions of LoVo cells transiently transfected with a $PC1^{WT}$ or a $PC1^{G483R}$ expression vector, using an anti-PC1-CT antibody (upper panel) or an anti-β actin antibody (lower panel). $PC1^{G483R}$ is present in the nuclear fraction of LoVo cells. Actin was used as a negative control for nuclear fractionation. Experiment was repeated once with similar results. T, total homogenates; C, cytoplasmic fraction; N, nuclear fraction.


A
HEK293
LoVo
a
c
PC1wt
b
d
PC1G483R

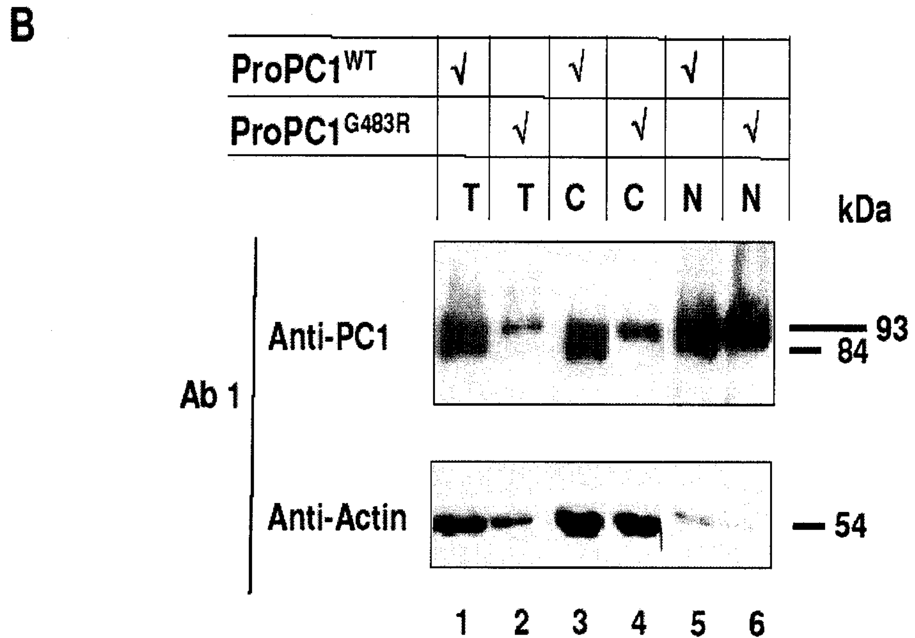
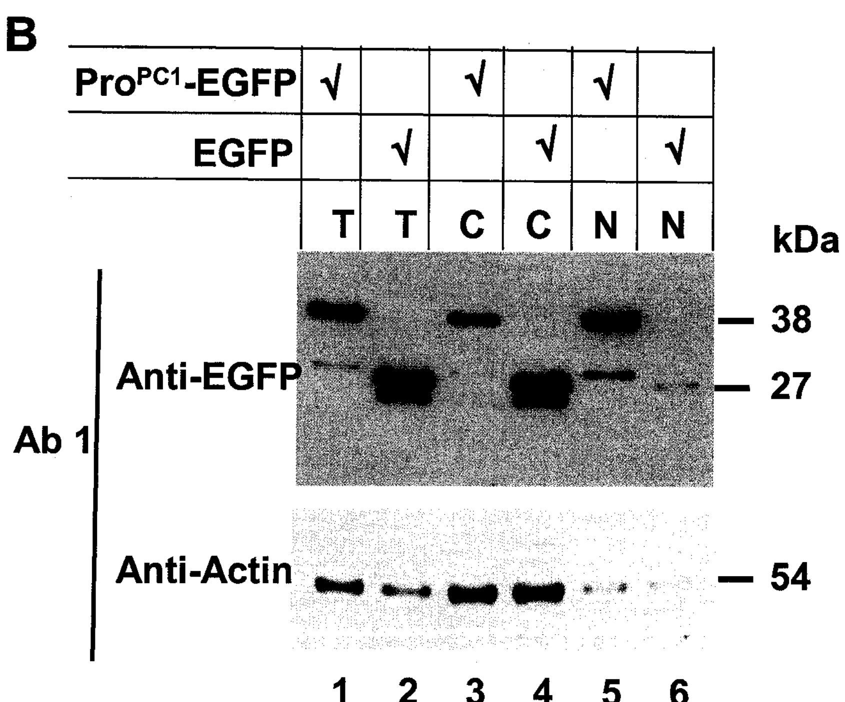

B
ProPC1WT
ProPC1G483R
T
T
C
C
N
N
kDa
Ab 1
Anti-PC1
93
84
Anti-Actin
54
1
2
3
4
5
6

FIGURE 11. *The prodomain of PC1 contains a putative NLS.* Transient transduction with Prepro^{PC1}-EGFP and EGFP in LoVo cells. **(A)** Sequence of the preprodomain of human PC1. The pre domain (signal peptide) is highlighted with a dashed overline. The putative NLS with the 4-residue pattern shown in bold is boxed. The junction between the preprodomain and the EGFP sequences is highlighted with a double underline. **(B)** Immunoblot of transfected cell subfractions for EGFP (upper panel) or an anti-β actin (lower panel). Pro^{PC1}-EGFP is expressed in the nuclear fraction of LoVo cells. Actin was used as a negative control for nuclear fractionation. Experiment was repeated once with similar results. T, total homogenates; C, cytoplasmic fraction; N, nuclear fraction.

A

MERRAWSLQCTAFVLFCAWCALNSAKAKRQF
VNEWAAEIPGGPEAASAIAEELGYDLLGQIG
SLENHYLFKHKNH**PRRSRR**SAFHITKRLSDD
DRVIWAEQQYEKERSKRSALRDSDPPVATMV
SKGEEL..egfp



4.0 DISCUSSION

4.1 Proprotein Convertases are Implicated in Embryonic Development

PCs have been clearly implicated in post-implantation development. In mouse, PACE4 has been shown to be necessary for the maturation of the precursor for Nodal and other bone morphogenetic proteins regulating the antero-posterior and the left/right patterning in early embryos (Constam and Robertson, 2000a). In zebrafish, a furin-like convertase mediates the processing of the gastrulation mitogen GPC-3 (De Cat et al., 2003). PC1 transcripts are reportedly found in the neural fold of *Xenopus laevis* embryos (Holling et al., 2000). To our knowledge, expression of PCs during mammalian preimplantation embryo development has never been studied. Several PC substrates are expressed at these developmental stages (see Section 1.11.2.).

Previously our lab showed that transcripts for all the PCs, except for PC2, are present in eggs and in preimplantation embryos at every developmental stage. Correspondingly, transcripts for 7B2, the PC2-specific chaperone and transient inhibitor, were undetectable. Unlike transcripts for other PCs which either decrease or remain unchanged, PC1 transcripts appear to increase as the fertilized eggs develop to blastocysts, suggesting that its expression might be up-regulated. Interestingly, transcripts for SAAS, the PC1-specific inhibitor, were detectable mostly in blastocysts where the 66-kDa PC1 may be the predominant form, suggesting that SAAS modulation of PC1 activity occurs primarily at this stage. The levels of CPE transcripts and proteins remain comparable at all stages. Taken together, these results indicate that the various components of the prohormone/proneuropeptide conversion system are present and functional in preimplantation embryos.

4.2. Nuclear Endoproteinases: Mechanism of Translocation and Potential Roles

The finding that PC1 immunoreactivity translocates into the pronuclei of fertilized eggs was most surprising. No PC has ever been reported to traffic to the nucleus. Several endoproteinases have been reported to be present in the nucleus, among them a calcium-dependent serine protease that cleaves the lamins A/C intermediate filaments of the nuclear scaffold (Clawson et al., 1992), caspase 1 and caspase 2 (Colussi et al., 1998; Mao et al., 1998).

Targeting of caspases 1 and 2 to the nucleus is reportedly directed by an NLS motif within their respective prodomain (Colussi et al., 1998; Mao et al., 1998). Likewise, it appears that the prodomain of PC1 exhibits a dominant nuclear targeting property since it can target a heterologous cytoplasmic protein to the nucleus. The secondary cleavage site within the prodomain of PC1 (KHKSHPRRSRR) (see Figures 4 and 11) is basic residue-rich and meets the criteria for a monopartite NLS (PredictNLS algorithm at http://cubic.bioc.columbia.edu/predictNLS) (Cokol et al., 2000). The PRRSRR segment corresponds to the pat7 NLS motif defined by the presence of a P residue followed by a basic segment containing 3 basic residues out of 4 (Hicks and Raikhel, 1995). Whether this motif is indeed an active NLS will eventually be determined by structure-function analyses using the prepro^{PC1}-EGFP variants in which the motif has been mutagenized.

PC1 in fertilized eggs is most likely to be of maternal origin as general transcription of zygotic genes begins at the 2-cell stage (Bouniol et al., 1995; Ma et al., 2001). One can assume that, like in somatic cells, this enzyme is contained within

secretory vesicles. One can further speculate that its translocation to the pronuclei results from the cytoskeletal-mediated membrane fusion between these organelles around the decondensing chromatin to form the nuclear envelope. Formation of such an envelope around the sea urchin male pronucleus has been shown to involve the recruitment and fusion of egg membrane vesicles (Collas, 2000).

It is still unclear with what nuclear components PC1 interacts or whether this interaction is enzymatic in nature. The term 'moonlighting' has been used to describe proteins with multiple functions (Jeffery, 1999). Although PC1 is defined by its ability to specifically process substrates at pairs of basic residues as part of the secretory pathway, the observation that PC1 translocates to the nucleus in fertilized mouse embryos may point to a potential moonlighting function for the convertase. Procorticotropin-releasing hormone, a hypothalamic prohormone, has been shown to translocate to the nucleus when transduced in the Chinese hamster ovary CHO-K1 cells and to strongly attach to chromatin (Morrison et al., 1995). Chromatin immunoprecipitation (Chip) technique (Kang et al., 2002; Kuo and Allis, 1999) could be used to determine whether PC1 also attaches to chromatin and at what specific genomic regions. This technique may elucidate a moonlighting function of PC1 involving transcriptional regulation at the preimplantation level. If activated and under appropriate pH and calcium conditions, nuclear PC1 may regulate gene expression by proteolytic processing of nuclear proteins (e.g. histones, transcription factors) after pairs of basic residues. The sequence of many nuclear proteins contains clusters of basic residues, especially within their NLS motifs (Cokol et al., 2000). Sperm histone processing by a cysteine protease during pronucleus formation has been described in sea urchin (Imschenetzky et al., 1997).

4.3 *Significance of PC1 Association with Membrane*

After the first cell division, PC1 is predominantly found in the cytoplasm and concentrated at intercellular junctions, especially at 2-cell embryos, in morula and in blastocysts. In blastocysts, the C-terminus-intact forms of PC1 are primarily localized at the periphery of blastomeres. It has been recently reported that a fraction of PC1 can reside as integral membrane protein in lipid raft-like structure of neuroendocrine secretory granules (Arnaoutova et al., 2003; Blazquez et al., 2001). Lipid rafts are cholesterol and sphingolipid-rich protein complexes found at the plasma membrane where they serve as signal transduction platforms (Simons and Toomre, 2000). The possible presence of PC1 in such complexes in preimplantation embryos suggests that, besides processing proproteins in the secretory pathway, PC1 may influence plasma membrane events, including intercellular adhesion and communication.

4.4 *Can our Findings Explain the Phenotype of PC1Δ30 Mice?*

This study was initiated following the observation made in our laboratory that PC1-null embryos homozygous for a 30 kDa deletion at the PCSK1 die before the blastocyst stage. Following the findings that PC1 is expressed during preimplantation embryonic development and translocates to the pronuclei of fertilized eggs via its prodomain, we re-examined the gene construct used to inactivate the PCSK1 locus in mouse embryonic stem cells from which the PC1Δ30 mice were generated. The construct is shown in Figure 12A. The PC1 gene promoter in this construct could still be active and drive the expression of a truncated prepro PC1 of 91 amino acids terminating a few residues after the putative NLS in the prodomain (Figure 12B). It will be

interesting to determine in a cell line transfected with the construct whether such a protein can be produced and whether it translocates from the cytoplasm to the nucleus.

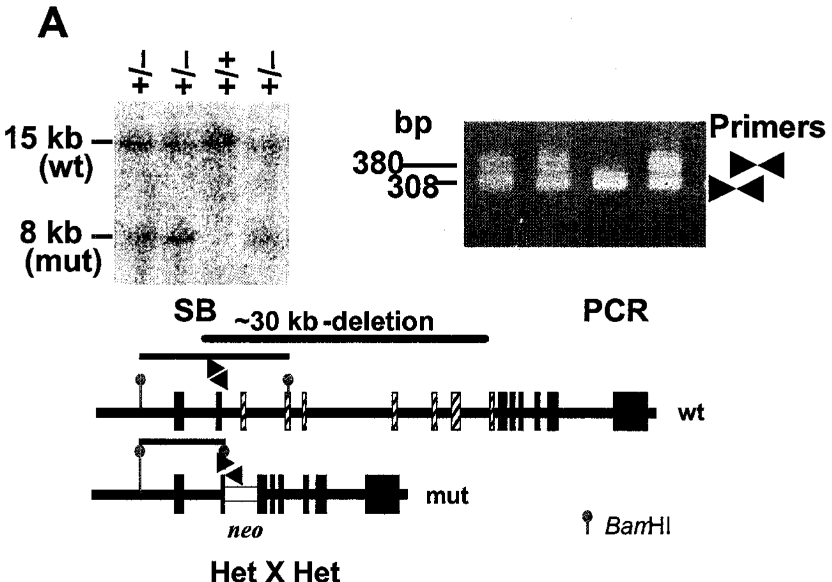
The existence of this truncated PC1 propeptide in the embryos may have other consequences on the physiology of preimplantation embryos. It is possible that such a peptide might act as a broad PC inhibitor during development. In vitro studies have shown that peptides from PC prodomains can not only inhibit the cognate inhibitor of the PC from which it was derived, but also, to a significant extent, other members of the family. N-terminal propeptides containing residues 1-84 of the proregion can act as potent slow tight-binding inhibitors to mPC1 and hfurin with K_is in the low nanomolar range (Boudreault et al., 1998b). $PC1^{50-83}$ propeptide has been shown to inhibit both PC1 and furin with a K_i of 0.7 and 4.8 μM, respectively (Basak and Lazure, 2003). Interestingly, a $PC1^{39-62}$ propeptide which contains our predicted NLS segment not only inhibited its cognate enzyme (K_i 15.4 μM) but was a much better inhibitor of hfurin (K_i 0.7 μM). Similarly, Fugère et al. (Fugere et al., 2002) showed that a short PC1 propeptide is capable of inhibiting not only its cognate enzyme (K_i, 184 nm) but PC5 as well and more potently (K_i 27 nm). The lack of specificity was attributed to the lack of specificity of the determinants, to the secondary binding sites essential for specificity, and to the stunted structure of the produced peptide which is not sufficient to conform to the enzyme subsites.

The truncated proPC1 expressed by the disrupted allele may exhibit a similar lack of specificity, inhibiting all the embryonic PCs and leading to a generalized impairment of endoproteolytic activation of precursors to peptides and proteins necessary for embryo survival.

The transmission ratio distortion of the *Pcsk1* allele among the offspring of heterozygous intercrosses (Figure 12C) can tentatively be explained by this inhibitory activity. The distortion is probably due to preferential survival of heterozygous embryos in the oviduct. In wild-type mice, the normal level of convertase activities allows balanced production of growth-promoting and growth-inhibiting factors. Assuming that precursors for growth-promoting factors are better PC substrates, the severely limiting level of convertase activity in heterozygous embryos may cause a preferential activation of growth-promoting factors, giving these mutant embryos survival advantages over wild type embryos. This speculative model remains to be tested.

FIGURE 12. *Disrupted Pcskl Allele and its Expressed Protein. Production of Pcskl+/del30 Mice.* The deletion was introduced into ES cells by homologous recombination. Chimera were produced from these cells and used to generate the Pcsk1+/del30. Heterozygosity for the deletion was confirmed by Southern blot (SB) analysis or PCR on genomic DNA. A diagrammatic representation of the wild type and mutant alleles is shown below (**A**). The amino acid sequence of the truncated protein produced from *Pcskl+/del30*. Green represents the signal peptide sequence. Red highlights the NLS sequence within the pro domain (**B**). The transmission ratio distortion of the *Pcskl* allele among the offspring of heterozygous intercrosses. W, homozygous wild type; D, heterozygote. (**C**)

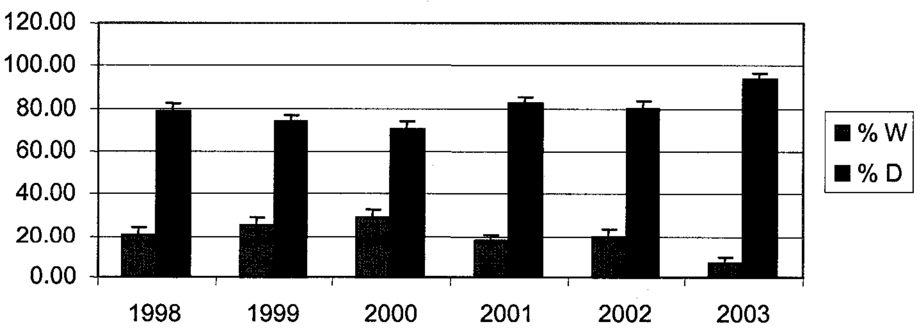
A



B

MEQRGWTLQCTAFAFFCVWCALNSVKAKRQFVNE
WAAEIPGGQEAASAIAEELGYDLLGIGSLENHYL
FKHKSHPRRSRRSALHGSVNSTG

C



5.0 FUTURE WORK

We have found that PC1 is expressed in preimplantation embryos and is transiently transported to pronuclei at the 1-cell stage. These findings raise a number of questions about both the mechanism of its trafficking and its cellular roles. We have speculated that this translocation results from the cytoskeletal-mediated membrane fusion between PC1-containing egg vesicles organelles around the decondensing chromatin to form the nuclear envelope.

To further characterize the molecular basis of the nuclear translocation, recombinant prepro^{PC1}-EGFP could be injected into pronuclear stage embryos and expression and translocation of reporter protein monitored in real-time. In addition, it should be also possible to determine in the same manner whether the predicted monopartite NLS (KHKSHPRRSRR) is indeed active using prepro^{PC1}-EGFP variants in which the motif has been mutagenized. If chromatin immunoprecipitation (Chip) (Kang et al., 2002; Kuo and Allis, 1999) assays indicate that PC1 can attach to chromatin, the genomic regions involved can be identified by PCR. To establish whether PC1 enzymatic activity in the nucleus is required for normal development, one can inject a proSAAS variant carrying a NLS into pronuclear stage embryos and examine the development of the embryos in culture.

To assess the functional relevance of PC1, one can culture fertilized eggs in the presence of a cell-permeant SAAS dodecapeptide (available from Dr. A. Basak, Ottawa Health Research Institute) and determine their rate of development to the blastocyst stage. Titration of known substrates such as proGnRH in the culture medium using antibodies specific for the processed form might confirm the inhibition of PC1 by the permeant SAAS peptide.

Finally, the hypothesis that a truncated inhibitory PC1 propeptide is produced in $PC1^{del30}$ heterozygous embryos could be verified by RT-PCR of the corresponding mRNA from a pool of blastocysts generated by heterozygote intercrosses. Because of the transmission ratio distortion, heterozygotes will constitute the majority of blastocysts. Specific primers can be designed for this implication since the 3'-UTR of the truncated mRNA is predictably different from that of native PC1 mRNA. The protein should be detected by immunoblotting using an antibody specific for the propeptide of PC1 (a gift from Dr. C. Lazure, Clinical Research Institute of Montreal). The generalized inhibition of convertases can be evaluated by verifying the processing of proGnRH (a PC1 substrate), proIGF-1R (a furin substrate) and pro-integrin αv (a PC5 and furin substrate).

6.0 CONCLUSIONS

This study demonstrates for the first time that PC1 protein forms are found in the mouse oocyte and preimplantation embryos. It also reports the curious observation that PC1 transiently translocates to the pronuclei of fertilized eggs and accumulates at intercellular junctions in post-zygotic embryos. This is a novel finding which goes against the established notion that the convertases are directed to the compartments of the secretory pathway exclusively. A nuclear localization signal localized within the prodomain of PC1 appears to be responsible for this translocation. The physiological relevance of PC1 expression and trafficking during preimplantation development remains to be clarified.

7.0 REFERENCES

Abrami, L., Fivaz, M., Decroly, E., Seidah, N. G., Jean, F., Thomas, G., Leppla, S. H., Buckley, J. T., and van der Goot, F. G. (1998). The pore-forming toxin proaerolysin is activated by furin. *J Biol Chem* **273,** 32656-32661.

Anderson, E. D., Molloy, S. S., Jean, F., Fei, H., Shimamura, S., and Thomas, G. (2002). The ordered and compartment-specfific autoproteolytic removal of the furin intramolecular chaperone is required for enzyme activation. *J Biol Chem* **277,** 12879-12890.

Arnaoutova, I., Smith, A. M., Coates, L. C., Sharpe, J. C., Dhanvantari, S., Snell, C. R., Birch, N. P., and Loh, Y. P. (2003). The prohormone processing enzyme PC3 is a lipid raft-associated transmembrane protein. *Biochemistry* **42,** 10445-10455.

Ayoubi, T. A., Creemers, J. W., Roebroek, A. J., and Van de Ven, W. J. (1994). Expression of the dibasic proprotein processing enzyme furin is directed by multiple promoters. *J Biol Chem* **269,** 9298-9303.

Barr, P. J., Mason, O. B., Landsberg, K. E., Wong, P. A., Kiefer, M. C., and Brake, A. J. (1991). cDNA and gene structure for a human subtilisin-like protease with cleavage specificity for paired basic amino acid residues. *DNA Cell Biol* **10,** 319-328.

Basak, A., Koch, P., Dupelle, M., Fricker, L. D., Devi, L. A., Chretien, M., and Seidah, N. G. (2001). Inhibitory specificity and potency of proSAAS-derived peptides toward proprotein convertase 1. *J Biol Chem* **276,** 32720-32728.

Basak, A., and Lazure, C. (2003). Synthetic peptides derived from the prosegments of proprotein convertase 1/3 and furin are potent inhibitors of both enzymes. *Biochem J* **373,** 231-239.

Basak, A., Toure, B. B., Lazure, C., Mbikay, M., Chretien, M., and Seidah, N. G. (1999). Enzymic characterization in vitro of recombinant proprotein convertase PC4. *Biochem J* **343 Pt 1,** 29-37.

Basak, S., Chretien, M., Mbikay, M., and Basak, A. (2004). In vitro elucidation of substrate specificity and bioassay of proprotein convertase 4 using intramolecularly quenched fluorogenic peptides. *Biochem J* **380,** 505-514.

Bassi, D. E., Mahloogi, H., Lopez De Cicco, R., and Klein-Szanto, A. (2003). Increased furin activity enhances the malignant phenotype of human head and neck cancer cells. *Am J Pathol* **162,** 439-447.

Bathurst, I. C., Brennan, S. O., Carrell, R. W., Cousens, L. S., Brake, A. J., and Barr, P. J. (1987). Yeast KEX2 protease has the properties of a human proalbumin converting enzyme. *Science* **235,** 348-350.

Benjannet, S., Rondeau, N., Day, R., Chretien, M., and Seidah, N. G. (1991). PC1 and PC2 are proprotein convertases capable of cleaving proopiomelanocortin at distinct pairs of basic residues. *Proc Natl Acad Sci U S A* **88,** 3564-3568.

Benjannet, S., Rondeau, N., Paquet, L., Boudreault, A., Lazure, C., Chretien, M., and Seidah, N. G. (1993). Comparative biosynthesis, covalent post-translational modifications and efficiency of prosegment cleavage of the prohormone convertases PC1 and PC2: glycosylation, sulphation and identification of the intracellular site of prosegment cleavage of PC1 and PC2. *Biochem J* **294 (Pt 3),** 735-743.

Bergeron, E., Basak, A., Decroly, E., and Seidah, N. G. (2003). Processing of alpha4 integrin by the proprotein convertases: histidine at position P6 regulates cleavage. *Biochem J* **373,** 475-484.

Blazquez, M., Docherty, K., and Shennan, K. I. (2001). Association of prohormone convertase 3 with membrane lipid rafts. *J Mol Endocrinol* **27,** 107-116.

Bloor, D. J., Metcalfe, A. D., Rutherford, A., Brison, D. R., and Kimber, S. J. (2002). Expression of cell adhesion molecules during human preimplantation embryo development. *Mol Hum Reprod* **8,** 237-245.

Boudreault, A., Gauthier, D., and Lazure, C. (1998a). Proprotein convertase PC1/3-related peptides are potent slow tight-binding inhibitors of murine PC1/3 and Hfurin. *J Biol Chem* **273,** 31574-31580.

Boudreault, A., Gauthier, D., Rondeau, N., Savaria, D., Seidah, N. G., Chretien, M., and Lazure, C. (1998b). Molecular characterization, enzymatic analysis, and purification of murine proprotein convertase-1/3 (PC1/PC3) secreted from recombinant baculovirus-infected insect cells. *Protein Expr Purif* **14,** 353-366.

Bouniol, C., Nguyen, E., and Debey, P. (1995). Endogenous transcription occurs at the 1-cell stage in the mouse embryo. *Exp Cell Res* **218,** 57-62.

Bradbury, A. F., Smyth, D. G., and Snell, C. R. (1976). Prohormones of beta-melanotropin (beta-melanocyte-stimulating hormone, beta-MSH) and corticotropin (adrenocorticotropic hormone, ACTH): structure and activation. *Ciba Found Symp* **41,** 61-75.

Braks, J. A., Guldemond, K. C., van Riel, M. C., Coenen, A. J., and Martens, G. J. (1992). Structure and expression of Xenopus prohormone convertase PC2. *FEBS Lett* **305,** 45-50.

Brenner, C. (2003). Subtleties among subtilases. The structural biology of Kex2 and furin-related prohormone convertases. *EMBO Rep* **4,** 937-938.

Bresnahan, P. A., Leduc, R., Thomas, L., Thorner, J., Gibson, H. L., Brake, A. J., Barr, P. J., and Thomas, G. (1990). Human fur gene encodes a yeast KEX2-like endoprotease that cleaves pro-beta-NGF in vivo. *J Cell Biol* **111,** 2851-2859.

Bruzzaniti, A., Goodge, K., Jay, P., Taviaux, S. A., Lam, M. H., Berta, P., Martin, T. J., Moseley, J. M., and Gillespie, M. T. (1996). PC8, a new member of the convertase family. *Biochem J* **314 (Pt 3),** 727-731.

Cain, B. M., Connolly, K., Blum, A. C., Vishnuvardhan, D., Marchand, J. E., Zhu, X., Steiner, D. F., and Beinfeld, M. C. (2004). Genetic inactivation of prohormone convertase (PC1) causes a reduction in cholecystokinin (CCK) levels in the hippocampus, amygdala, pons and medulla in mouse brain that correlates with the degree of colocalization of PC1 and CCK mRNA in these structures in rat brain. *J Neurochem* **89,** 307-313.

Campbell, S., Swann, H. R., Seif, M. W., Kimber, S. J., and Aplin, J. D. (1995). Cell adhesion molecules on the oocyte and preimplantation human embryo. *Hum Reprod* **10,** 1571-1578.

Casan, E. M., Raga, F., and Polan, M. L. (1999). GnRH mRNA and protein expression in human preimplantation embryos. *Mol Hum Reprod* **5,** 234-239.

Chan, S. J., Oliva, A. A., Jr., LaMendola, J., Grens, A., Bode, H., and Steiner, D. F. (1992). Conservation of the prohormone convertase gene family in metazoa: analysis of cDNAs encoding a PC3-like protein from hydra. *Proc Natl Acad Sci U S A* **89,** 6678-6682.

Chretien, M., Gossard, F., Crine, P., Gianoulakis, C., and Seidah, N. G. (1980). New perspectives in neuropeptides. Biosynthesis from large precursors. The pro-opio-melanocortin model in the pars intermedia. *Acta Psychiatr Belg* **80,** 699-713.

Chretien, M., and Li, C. H. (1967). Isolation, purification, and characterization of gamma-lipotropic hormone from sheep pituitary glands. *Can J Biochem* **45,** 1163-1174.

Chretien, M., Mbikay, M., Gaspar, L., and Seidah, N. G. (1995). Proprotein convertases and the pathophysiology of human diseases: prospective considerations. *Proc Assoc Am Physicians* **107,** 47-66.

Cieplik, M., Klenk, H. D., and Garten, W. (1998). Identification and characterization of spodoptera frugiperda furin: a thermostable subtilisin-like endopeptidase. *Biol Chem* **379,** 1433-1440.

Clawson, G. A., Norbeck, L. L., Hatem, C. L., Rhodes, C., Amiri, P., McKerrow, J. H., Patierno, S. R., and Fiskum, G. (1992). Ca(2+)-regulated serine protease associated with the nuclear scaffold. *Cell Growth Differ* **3,** 827-838.

Coates, L. C., and Birch, N. P. (1998). Differential cleavage of provasopressin by the major molecular forms of SPC3. *J Neurochem* **70,** 1670-1678.

Cokol, M., Nair, R., and Rost, B. (2000). Finding nuclear localization signals. *EMBO Rep* **1,** 411-415.

Collas, P. (2000). Formation of the sea urchin male pronucleus in cell-free extracts. *Mol Reprod Dev* **56,** 265-270.

Colussi, P. A., Harvey, N. L., and Kumar, S. (1998). Prodomain-dependent nuclear localization of the caspase-2 (Nedd2) precursor. A novel function for a caspase prodomain. *J Biol Chem* **273,** 24535-24542.

Constam, D. B., and Robertson, E. J. (2000a). SPC4/PACE4 regulates a TGFbeta signaling network during axis formation. *Genes Dev* **14,** 1146-1155.

Constam, D. B., and Robertson, E. J. (2000b). Tissue-specific requirements for the proprotein convertase furin/SPC1 during embryonic turning and heart looping. *Development* **127,** 245-254.

Copeland, N. G., Gilbert, D. J., Chretien, M., Seidah, N. G., and Jenkins, N. A. (1992). Regional localization of three convertases, PC1 (Nec-1), PC2 (Nec-2), and furin (Fur), on mouse chromosomes. *Genomics* **13,** 1356-1358.

Croissandeau, G., Basak, A., Seidah, N. G., Chretien, M., and Mbikay, M. (2002). Proprotein convertases are important mediators of the adipocyte differentiation of mouse 3T3-L1 cells. *J Cell Sci* **115,** 1203-1211.

Day, R., Schafer, M. K., Cullinan, W. E., Watson, S. J., Chretien, M., and Seidah, N. G. (1993). Region specific expression of furin mRNA in the rat brain. *Neurosci Lett* **149,** 27-30.

De Bie, I., Marcinkiewicz, M., Malide, D., Lazure, C., Nakayama, K., Bendayan, M., and Seidah, N. G. (1996). The isoforms of proprotein convertase PC5 are sorted to different subcellular compartments. *J Cell Biol* **135,** 1261-1275.

De Cat, B., Muyldermans, S. Y., Coomans, C., Degeest, G., Vanderschueren, B., Creemers, J., Biemar, F., Peers, B., and David, G. (2003). Processing by proprotein convertases is required for glypican-3 modulation of cell survival, Wnt signaling, and gastrulation movements. *Journal of Cell Biology* **163,** 625-635.

Decroly, E., Wouters, S., Di Bello, C., Lazure, C., Ruysschaert, J. M., and Seidah, N. G. (1996). Identification of the paired basic convertases implicated in HIV gp160 processing based on in vitro assays and expression in CD4(+) cell lines. *J Biol Chem* **271,** 30442-30450.

Dhanvantari, S., Seidah, N. G., and Brubaker, P. L. (1996). Role of prohormone convertases in the tissue-specific processing of proglucagon. *Mol Endocrinol* **10,** 342-355.

Diaz-Cueto, L., and Gerton, G. L. (2001). The influence of growth factors on the development of preimplantation mammalian embryos. *Arch Med Res* **32,** 619-626.

Dong, W., Fricker, L. D., and Day, R. (1999). Carboxypeptidase D is a potential candidate to carry out redundant processing functions of carboxypeptidase E based on comparative distribution studies in the rat central nervous system. *Neuroscience* **89,** 1301-1317.

Dong, W., Marcinkiewicz, M., Vieau, D., Chretien, M., Seidah, N. G., and Day, R. (1995). Distinct mRNA expression of the highly homologous convertases PC5 and PACE4 in the rat brain and pituitary. *J Neurosci* **15,** 1778-1796.

Dubey, A. K., Cruz, J. R., Hartog, B., and Gindoff, P. R. (2001). Expression of the alphav integrin adhesion molecule during development of preimplantation human embryos. *Fertil Steril* **76,** 153-156.

Dubois, C. M., Blanchette, F., Laprise, M. H., Leduc, R., Grondin, F., and Seidah, N. G. (2001). Evidence that furin is an authentic transforming growth factor-beta1-converting enzyme. *Am J Pathol* **158,** 305-316.

Dubois, C. M., Laprise, M. H., Blanchette, F., Gentry, L. E., and Leduc, R. (1995). Processing of transforming growth factor beta 1 precursor by human furin convertase. *J Biol Chem* **270,** 10618-10624.

Duckert, P., Brunak, S., and Blom, N. (2004). Prediction of proprotein convertase cleavage sites. *Protein Eng Des Sel* **17,** 107-112.

Duguay, S. J., Lai-Zhang, J., and Steiner, D. F. (1995). Mutational analysis of the insulin-like growth factor I prohormone processing site. *J Biol Chem* **270,** 17566-17574.

Eipper, B. A., Green, C. B., and Mains, R. E. (1992). Expression of prohormone processing enzymes in neuroendocrine and non-neuroendocrine cells. *J Natl Cancer Inst Monogr*, 163-168.

Fricker, L. D. (1988). Carboxypeptidase E. *Annu Rev Physiol* **50,** 309-321.

Fricker, L. D., and Leiter, E. H. (1999). Peptides, enzymes and obesity: new insights from a 'dead' enzyme. *Trends Biochem Sci* **24,** 390-393.

Fricker, L. D., McKinzie, A. A., Sun, J., Curran, E., Qian, Y., Yan, L., Patterson, S. D., Courchesne, P. L., Richards, B., Levin, N., Mzhavia, N., Devi, L. A., and Douglass, J. (2000). Identification and characterization of proSAAS, a granin-like neuroendocrine peptide precursor that inhibits prohormone processing. *J Neurosci* **20,** 639-648.

Ftouhi, N., Day, R., Mbikay, M., Chretien, M., and Seidah, N. G. (1994). Gene organization of the mouse pro-hormone and pro-protein convertase PC1. *DNA Cell Biol* **13,** 395-407.

Fugere, M., Limperis, P. C., Beaulieu-Audy, V., Gagnon, F., Lavigne, P., Klarskov, K., Leduc, R., and Day, R. (2002). Inhibitory potency and specificity of subtilase-like pro-protein convertase (SPC) prodomains. *J Biol Chem* **277,** 7648-7656.

Fuller, R. S., Brake, A. J., and Thorner, J. (1989). Intracellular targeting and structural conservation of a prohormone-processing endoprotease. *Science* **246,** 482-486.

Furuta, M., Carroll, R., Martin, S., Swift, H. H., Ravazzola, M., Orci, L., and Steiner, D. F. (1998). Incomplete processing of proinsulin to insulin accompanied by elevation

of Des-31,32 proinsulin intermediates in islets of mice lacking active PC2. *J Biol Chem* **273,** 3431-3437.

Furuta, M., Yano, H., Zhou, A., Rouille, Y., Holst, J. J., Carroll, R., Ravazzola, M., Orci, L., Furuta, H., and Steiner, D. F. (1997). Defective prohormone processing and altered pancreatic islet morphology in mice lacking active SPC2. *Proc Natl Acad Sci U S A* **94,** 6646-6651.

Furuta, M., Zhou, A., Webb, G., Carroll, R., Ravazzola, M., Orci, L., and Steiner, D. F. (2001). Severe defect in proglucagon processing in islet A-cells of prohormone convertase 2 null mice. *J Biol Chem* **276,** 27197-27202.

Gainer, H., Russell, J. T., and Loh, Y. P. (1985). The enzymology and intracellular organization of peptide precursor processing: the secretory vesicle hypothesis. *Neuroendocrinology* **40,** 171-184.

Gangnon, F., Danger, J. M., Jegou, S., Vieau, D., Seidah, N. G., and Vaudry, H. (1999). Molecular cloning, characterization of cDNA, and distribution of mRNA encoding the frog prohormone convertase PC1. *J Comp Neurol* **405,** 160-172.

Gangnon, F., Jegou, S., Vallarino, M., Vieau, D., and Vaudry, H. (2003). Molecular characterization of the cDNA and localization of the mRNA encoding the prohormone convertase PC5-A in the European green frog. *J Comp Neurol* **456,** 60-72.

Gomez-Saladin, E., Wilson, D. L., and Dickerson, I. M. (1994). Isolation and in situ localization of a cDNA encoding a Kex2-like prohormone convertase in the nematode Caenorhabditis elegans. *Cell Mol Neurobiol* **14,** 9-25.

Goodge, K. A., Thomas, R. J., Martin, T. J., and Gillespie, M. T. (1998). Gene organization and alternative splicing of human prohormone convertase PC8. *Biochem J* **336 (Pt 2),** 353-359.

Gorham, E. L., Nagle, G. T., Smith, J. S., Shen, H., and Kurosky, A. (1996). Molecular cloning of prohormone convertase 1 from the atrial gland of Aplysia. *DNA Cell Biol* **15,** 339-345.

Gu, M., Gordon, V. M., Fitzgerald, D. J., and Leppla, S. H. (1996). Furin regulates both the activation of Pseudomonas exotoxin A and the Quantity of the toxin receptor expressed on target cells. *Infect Immun* **64,** 524-527.

Gyamera-Acheampong, C., Tantibhedhangakul, J., Tadros, H., Sirois, F., Weerachatyanukul, W., van de Loo, J. W., Pelletier, R. M., Tanphaichitr, N., and Mbikay, M. (2004). Mouse Proprotein Convertase 4 is located on the acrosomal plasma membrane and its deficiency renders spermatozoa less efficient at binding to egg zona pellucida. *Biology of Reproduction* **submitted.**

Haimovici, F., and Anderson, D. J. (1993). Effects of growth factors and growth factor-extracellular matrix interactions on mouse trophoblast outgrowth in vitro. *Biol Reprod* **49,** 124-130.

Hallenberger, S., Bosch, V., Angliker, H., Shaw, E., Klenk, H. D., and Garten, W. (1992). Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. *Nature* **360,** 358-361.

Harvey, M. B., and Kaye, P. L. (1988). Insulin stimulates protein synthesis in compacted mouse embryos. *Endocrinology* **122,** 1182-1184.

Harvey, M. B., and Kaye, P. L. (1991). IGF-2 receptors are first expressed at the 2-cell stage of mouse development. *Development* **111,** 1057-1060.

Hicks, G. R., and Raikhel, N. V. (1995). Protein import into the nucleus: an integrated view. *Annu Rev Cell Dev Biol* **11,** 155-188.

Hogan, B. e. a. (1994). "Manipulating the mouse embryo. A laboratory manual." Cold Spring Harbor Laboratory Press, New York.

Holling, T. M., van Herp, F., Durston, A. J., and Martens, G. J. (2000). Differential onset of expression of mRNAs encoding proopiomelanocortin, prohormone convertases 1 and 2, and granin family members during Xenopus laevis development. *Brain Res Mol Brain Res* **75,** 70-75.

Imschenetzky, M., Diaz, F., Montecino, M., Sierra, F., and Puchi, M. (1997). Identification of a cysteine protease responsible for degradation of sperm histones during male pronucleus remodeling in sea urchins. *J Cell Biochem* **67,** 304-315.

Itoh, Y., Tanaka, S., Takekoshi, S., Itoh, J., and Osamura, R. Y. (1996). Prohormone convertases (PC1/3 and PC2) in rat and human pancreas and islet cell tumors: subcellular immunohistochemical analysis. *Pathol Int* **46,** 726-737.

Jackson, R. S., Creemers, J. W., Farooqi, I. S., Raffin-Sanson, M. L., Varro, A., Dockray, G. J., Holst, J. J., Brubaker, P. L., Corvol, P., Polonsky, K. S., Ostrega, D., Becker, K. L., Bertagna, X., Hutton, J. C., White, A., Dattani, M. T., Hussain, K., Middleton, S. J., Nicole, T. M., Milla, P. J., Lindley, K. J., and O'Rahilly, S. (2003). Small-intestinal dysfunction accompanies the complex endocrinopathy of human proprotein convertase 1 deficiency. *J Clin Invest* **112,** 1550-1560.

Jackson, R. S., Creemers, J. W., Ohagi, S., Raffin-Sanson, M. L., Sanders, L., Montague, C. T., Hutton, J. C., and O'Rahilly, S. (1997). Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene. *Nat Genet* **16,** 303-306.

Jeffery, C. J. (1999). Moonlighting Proteins. *Trends Biochem Sci* **24**, 8-11.

Jones, B. G., Thomas, L., Molloy, S. S., Thulin, C. D., Fry, M. D., Walsh, K. A., and Thomas, G. (1995). Intracellular trafficking of furin is modulated by the phosphorylation state of a casein kinase II site in its cytoplasmic tail. *Embo J* **14,** 5869-5883.

Julius, D., Brake, A., Blair, L., Kunisawa, R., and Thorner, J. (1984). Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-alpha-factor. *Cell* **37,** 1075-1089.

Kang, S. H., Vieira, K., and Bungert, J. (2002). Combining chromatin immunoprecipitation and DNA footprinting: a novel method to analyze protein-DNA interactions in vivo. *Nucleic Acids Res* **30,** e44.

Kayo, T., Konda, Y., Tanaka, S., Takata, K., Koizumi, A., and Takeuchi, T. (1996). Developmental expression of proprotein-processing endoprotease furin in rat pancreatic islets. *Endocrinology* **137,** 5126-5134.

Khatib, A. M., Siegfried, G., Prat, A., Luis, J., Chretien, M., Metrakos, P., and Seidah, N. G. (2001). Inhibition of proprotein convertases is associated with loss of growth and tumorigenicity of HT-29 human colon carcinoma cells: importance of insulin-like growth factor-1 (IGF-1) receptor processing in IGF-1-mediated functions. *J Biol Chem* **276,** 30686-30693.

Kiefer, M. C., Tucker, J. E., Joh, R., Landsberg, K. E., Saltman, D., and Barr, P. J. (1991). Identification of a second human subtilisin-like protease gene in the fes/fps region of chromosome 15. *DNA Cell Biol* **10,** 757-769.

Kimber, S. J., and Spanswick, C. (2000). Blastocyst implantation: the adhesion cascade. *Semin Cell Dev Biol* **11,** 77-92.

Kirchmair, R., Egger, C., Gee, P., Hogue-Angeletti, R., Fischer-Colbrie, R., Laslop, A., and Winkler, H. (1992). Differential subcellular distribution of PC1, PC2 and furin in bovine adrenal medulla and secretion of PC1 and PC2 from this tissue. *Neurosci Lett* **143,** 143-145.

Konda, Y., Yokota, H., Kayo, T., Horiuchi, T., Sugiyama, N., Tanaka, S., Takata, K., and Takeuchi, T. (1997). Proprotein-processing endoprotease furin controls the growth and differentiation of gastric surface mucous cells. *J Clin Invest* **99,** 1842-1851.

Kovaleva, E. S., Yakovlev, A. G., Masler, E. P., and Chitwood, D. J. (2002). Human proprotein convertase 2 homologue from a plant nematode: cloning, characterization, and comparison with other species. *Faseb J* **16,** 1099-1101.

Kuo, M. H., and Allis, C. D. (1999). In vivo cross-linking and immunoprecipitation for studying dynamic Protein:DNA associations in a chromatin environment. *Methods* **19,** 425-433.

Kurabuchi, S., and Tanaka, S. (2002). Immunocytochemical localization of prohormone convertases PC1 and PC2 in the mouse thyroid gland and respiratory tract. *J Histochem Cytochem* **50,** 903-909.

Laurent, V., Jaubert-Miazza, L., Desjardins, R., Day, R., and Lindberg, I. (2004). Biosynthesis of proopiomelanocortin-derived peptides in prohormone convertase 2 and 7B2 null mice. *Endocrinology* **145,** 519-528.

Lawitts, J. A., and Biggers, J. D. (1993). Culture of preimplantation embryos. *Methods Enzymol* **225,** 153-164.

Lazure, C., Seidah, N. G., Pelaprat, D., and Chretien, M. (1983). Proteases and posttranslational processing of prohormones: a review. *Can J Biochem Cell Biol* **61,** 501-515.

Lehmann, M., Andre, F., Bellan, C., Remacle-Bonnet, M., Garrouste, F., Parat, F., Lissitsky, J. C., Marvaldi, J., and Pommier, G. (1998). Deficient processing and activity of type I insulin-like growth factor receptor in the furin-deficient LoVo-C5 cells. *Endocrinology* **139,** 3763-3771.

Li, M., Mbikay, M., and Arimura, A. (2000). Pituitary adenylate cyclase-activating polypeptide precursor is processed solely by prohormone convertase 4 in the gonads. *Endocrinology* **141,** 3723-3730.

Lissitzky, J. C., Luis, J., Munzer, J. S., Benjannet, S., Parat, F., Chretien, M., Marvaldi, J., and Seidah, N. G. (2000). Endoproteolytic processing of integrin pro-alpha subunits involves the redundant function of furin and proprotein convertase (PC) 5A, but not paired basic amino acid converting enzyme (PACE) 4, PC5B or PC7. *Biochem J* **346 Pt 1,** 133-138.

Lusson, J., Vieau, D., Hamelin, J., Day, R., Chretien, M., and Seidah, N. G. (1993). cDNA structure of the mouse and rat subtilisin/kexin-like PC5: a candidate proprotein convertase expressed in endocrine and nonendocrine cells. *Proc Natl Acad Sci U S A* **90,** 6691-6695.

Ma, J., Svoboda, P., Schultz, R. M., and Stein, P. (2001). Regulation of zygotic gene activation in the preimplantation mouse embryo: global activation and repression of gene expression. *Biol Reprod* **64,** 1713-1721.

Malide, D., Seidah, N. G., Chretien, M., and Bendayan, M. (1995). Electron microscopic immunocytochemical evidence for the involvement of the convertases PC1 and

PC2 in the processing of proinsulin in pancreatic beta-cells. *J Histochem Cytochem* **43,** 11-19.

Mao, P. L., Jiang, Y., Wee, B. Y., and Porter, A. G. (1998). Activation of caspase-1 in the nucleus requires nuclear translocation of pro-caspase-1 mediated by its prodomain. *J Biol Chem* **273,** 23621-23624.

Marcinkiewicz, M., Day, R., Seidah, N. G., and Chretien, M. (1993). Ontogeny of the prohormone convertases PC1 and PC2 in the mouse hypophysis and their colocalization with corticotropin and alpha-melanotropin. *Proc Natl Acad Sci U S A* **90,** 4922-4926.

Marcinkiewicz, M., Ramla, D., Seidah, N. G., and Chretien, M. (1994). Developmental expression of the prohormone convertases PC1 and PC2 in mouse pancreatic islets. *Endocrinology* **135,** 1651-1660.

Mbikay, M., Raffin-Sanson, M. L., Tadros, H., Sirois, F., Seidah, N. G., and Chretien, M. (1994). Structure of the gene for the testis-specific proprotein convertase 4 and of its alternate messenger RNA isoforms. *Genomics* **20,** 231-237.

Mbikay, M., Seidah, N. G., and Chretien, M. (1993). From proopiomelanocortin to cancer. Possible role of convertases in neoplasia. *Ann N Y Acad Sci* **680,** 13-19.

Mbikay, M., Seidah, N. G., and Chretien, M. (2001). Neuroendocrine secretory protein 7B2: structure, expression and functions. *Biochem J* **357,** 329-342.

Mbikay, M., Seidah, N. G., Chretien, M., and Simpson, E. M. (1995). Chromosomal assignment of the genes for proprotein convertases PC4, PC5, and PACE 4 in mouse and human. *Genomics* **26,** 123-129.

Mbikay, M., Tadros, H., Ishida, N., Lerner, C. P., De Lamirande, E., Chen, A., El-Alfy, M., Clermont, Y., Seidah, N. G., Chretien, M., Gagnon, C., and Simpson, E. M.

(1997). Impaired fertility in mice deficient for the testicular germ-cell protease PC4. *Proc Natl Acad Sci U S A* **94,** 6842-6846.

Meerabux, J., Yaspo, M. L., Roebroek, A. J., Van de Ven, W. J., Lister, T. A., and Young, B. D. (1996). A new member of the proprotein convertase gene family (LPC) is located at a chromosome translocation breakpoint in lymphomas. *Cancer Res* **56,** 448-451.

Mentrup, B., Londershausen, M., Spindler, K., and Weidemann, W. (1999). Isolation and characterization of insect PC2-like prohormone convertase cDNA. *Insect Mol Biol* **8,** 305-310.

Mentrup, B., and Weidemann, W. (1999). The exon-intron organization of the prohormone convertase PC2 gene from the insect Lucilia cuprina. *Gene* **237,** 29-33.

Misumi, Y., Oda, K., Fujiwara, T., Takami, N., Tashiro, K., and Ikehara, Y. (1991). Functional expression of furin demonstrating its intracellular localization and endoprotease activity for processing of proalbumin and complement pro-C3. *J Biol Chem* **266,** 16954-16959.

Molloy, S. S., Bresnahan, P. A., Leppla, S. H., Klimpel, K. R., and Thomas, G. (1992). Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen. *J Biol Chem* **267,** 16396-16402.

Molloy, S. S., Thomas, L., VanSlyke, J. K., Stenberg, P. E., and Thomas, G. (1994). Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface. *Embo J* **13,** 18-33.

Morrison, E., Tomasec, P., Linton, E. A., Lowry, P. J., Lowenstein, P. R., and Castro, M. G. (1995). Expression of biologically active procorticotrophin-releasing hormone (proCRH) in stably transfected CHO-K1 cells: characterization of nuclear proCRH. *J Neuroendocrinol* **7,** 263-272.

Moulard, M., and Decroly, E. (2000). Maturation of HIV envelope glycoprotein precursors by cellular endoproteases. *Biochim Biophys Acta* **1469,** 121-132.

Muller, L., and Lindberg, I. (1999). The cell biology of the prohormone convertases PC1 and PC2. *Prog Nucleic Acid Res Mol Biol* **63,** 69-108.

Muller, L., Picart, R., Barret, A., Seidah, N. G., and Tougard, C. (1998). Immunocytochemical localization of the prohormone convertases PC1 and PC2 in rat prolactin cells. *J Histochem Cytochem* **46,** 101-108.

Murphy, L. J., and Barron, D. J. (1993). The IGFs and their binding proteins in murine development. *Mol Reprod Dev* **35,** 376-381.

Nagle, G. T., Garcia, A. T., Gorham, E. L., Knock, S. L., van Heumen, W. R., Spijker, S., Smit, A. B., Geraerts, W. P., and Kurosky, A. (1995a). Molecular cloning and cellular localization of a furin-like prohormone convertase from the atrial gland of Aplysia. *DNA Cell Biol* **14,** 431-443.

Nagle, G. T., Garcia, A. T., Knock, S. L., Gorham, E. L., Van Heumen, W. R., and Kurosky, A. (1995b). Molecular cloning, cDNA sequence, and localization of a prohormone convertase (PC2) from the Aplysia atrial gland. *DNA Cell Biol* **14,** 145-154.

Nakagawa, T., Hosaka, M., Torii, S., Watanabe, T., Murakami, K., and Nakayama, K. (1993). Identification and functional expression of a new member of the

mammalian Kex2-like processing endoprotease family: its striking structural similarity to PACE4. *J Biochem (Tokyo)* **113,** 132-135.

Nakayama, K., Kim, W. S., Torii, S., Hosaka, M., Nakagawa, T., Ikemizu, J., Baba, T., and Murakami, K. (1992). Identification of the fourth member of the mammalian endoprotease family homologous to the yeast Kex2 protease. Its testis-specific expression. *J Biol Chem* **267,** 5897-5900.

Neurath, H., and Walsh, K. A. (1976). Role of proteolytic enzymes in biological regulation (a review). *Proc Natl Acad Sci U S A* **73,** 3825-3832.

Nour, N., Basak, A., Chretien, M., and Seidah, N. G. (2003). Structure-function analysis of the prosegment of the proprotein convertase PC5A. *J Biol Chem* **278,** 2886-2895.

Ohagi, S., LaMendola, J., LeBeau, M. M., Espinosa, R., 3rd, Takeda, J., Smeekens, S. P., Chan, S. J., and Steiner, D. F. (1992). Identification and analysis of the gene encoding human PC2, a prohormone convertase expressed in neuroendocrine tissues. *Proc Natl Acad Sci U S A* **89,** 4977-4981.

Oliva, A. A., Jr., Chan, S. J., and Steiner, D. F. (2000). Evolution of the prohormone convertases: identification of a homologue of PC6 in the protochordate amphioxus. *Biochim Biophys Acta* **1477,** 338-348.

O'Neill, C. (1997). Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos in vitro. *Biol Reprod* **56,** 229-237.

Ouimet, T., Mammarbachi, A., Cloutier, T., Seidah, N. G., and Castellucci, V. F. (1993). cDNA structure and in situ localization of the Aplysia californica pro-hormone convertase PC2. *FEBS Lett* **330,** 343-346.

Palmieri, S. L., Payne, J., Stiles, C. D., Biggers, J. D., and Mercola, M. (1992). Expression of mouse PDGF-A and PDGF alpha-receptor genes during pre- and post-implantation development: evidence for a developmental shift from an autocrine to a paracrine mode of action. *Mech Dev* **39,** 181-191.

Pantaleon, M., and Kaye, P. L. (1996). IGF-I and insulin regulate glucose transport in mouse blastocysts via IGF-I receptor. *Mol Reprod Dev* **44,** 71-76.

Paria, B. C., Jones, K. L., Flanders, K. C., and Dey, S. K. (1992). Localization and binding of transforming growth factor-beta isoforms in mouse preimplantation embryos and in delayed and activated blastocysts. *Dev Biol* **151,** 91-104.

Poole, C. B., Jin, J., and McReynolds, L. A. (2003). Cloning and biochemical characterization of blisterase, a subtilisin-like convertase from the filarial parasite, Onchocerca volvulus. *J Biol Chem* **278,** 36183-36190.

Qian, Y., Devi, L. A., Mzhavia, N., Munzer, S., Seidah, N. G., and Fricker, L. D. (2000). The C-terminal region of proSAAS is a potent inhibitor of prohormone convertase 1. *Journal of Biological Chemistry* **275,** 23596-23601.

Raga, F., Casan, E. M., Kruessel, J., Wen, Y., Bonilla-Musoles, F., and Polan, M. L. (1999). The role of gonadotropin-releasing hormone in murine preimplantation embryonic development. *Endocrinology* **140,** 3705-3712.

Raga, F., Casan, E. M., Kruessel, J. S., Wen, Y., Huang, H. Y., Nezhat, C., and Polan, M. L. (1998). Quantitative gonadotropin-releasing hormone gene expression and immunohistochemical localization in human endometrium throughout the menstrual cycle. *Biol Reprod* **59,** 661-669.

Rangaraju, N. S., and Harris, R. B. (1993). GAP-releasing enzyme is a member of the pro-hormone convertase family of precursor protein processing enzymes. *Life Sci* **52,** 147-153.

Rappolee, D. A., Brenner, C. A., Schultz, R., Mark, D., and Werb, Z. (1988). Developmental expression of PDGF, TGF-alpha, and TGF-beta genes in preimplantation mouse embryos. *Science* **241,** 1823-1825.

Rehfeld, J. F., Lindberg, I., and Friis-Hansen, L. (2002). Increased synthesis but decreased processing of neuronal proCCK in prohormone convertase 2 and 7B2 knockout animals. *J Neurochem* **83,** 1329-1337.

Roebroek, A. J., Pauli, I. G., Zhang, Y., and van de Ven, W. J. (1991). cDNA sequence of a Drosophila melanogaster gene, Dfur1, encoding a protein structurally related to the subtilisin-like proprotein processing enzyme furin. *FEBS Lett* **289,** 133-137.

Roebroek, A. J., Schalken, J. A., Bussemakers, M. J., van Heerikhuizen, H., Onnekink, C., Debruyne, F. M., Bloemers, H. P., and Van de Ven, W. J. (1986a). Characterization of human c-fes/fps reveals a new transcription unit (fur) in the immediately upstream region of the proto-oncogene. *Mol Biol Rep* **11,** 117-125.

Roebroek, A. J., Schalken, J. A., Leunissen, J. A., Onnekink, C., Bloemers, H. P., and Van de Ven, W. J. (1986b). Evolutionary conserved close linkage of the c-fes/fps proto-oncogene and genetic sequences encoding a receptor-like protein. *Embo J* **5,** 2197-2202.

Roebroek, A. J., Umans, L., Pauli, I. G., Robertson, E. J., van Leuven, F., Van de Ven, W. J., and Constam, D. B. (1998). Failure of ventral closure and axial rotation in embryos lacking the proprotein convertase Furin. *Development* **125,** 4863-4876.

Roelen, B. A., Goumans, M. J., Zwijsen, A., and Mummery, C. L. (1998). Identification of two distinct functions for TGF-beta in early mouse development. *Differentiation* **64,** 19-31.

Sarac, M. S., Zieske, A. W., and Lindberg, I. (2002). The lethal form of Cushing's in 7B2 null mice is caused by multiple metabolic and hormonal abnormalities. *Endocrinology* **143,** 2324-2332.

Sayah, M., Fortenberry, Y., Cameron, A., and Lindberg, I. (2001). Tissue distribution and processing of proSAAS by proprotein convertases. *J Neurochem* **76,** 1833-1841.

Schafer, M. K., Day, R., Cullinan, W. E., Chretien, M., Seidah, N. G., and Watson, S. J. (1993). Gene expression of prohormone and proprotein convertases in the rat CNS: a comparative in situ hybridization analysis. *J Neurosci* **13,** 1258-1279.

Seidah, N. G., and Chretien, M. (1999). Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. *Brain Res* **848,** 45-62.

Seidah, N. G., Chretien, M., and Day, R. (1994). The family of subtilisin/kexin like pro-protein and pro-hormone convertases: divergent or shared functions. *Biochimie* **76,** 197-209.

Seidah, N. G., Day, R., Hamelin, J., Gaspar, A., Collard, M. W., and Chretien, M. (1992). Testicular expression of PC4 in the rat: molecular diversity of a novel germ cell-specific Kex2/subtilisin-like proprotein convertase. *Mol Endocrinol* **6,** 1559-1570.

Seidah, N. G., Hamelin, J., Mamarbachi, M., Dong, W., Tardos, H., Mbikay, M., Chretien, M., and Day, R. (1996). cDNA structure, tissue distribution, and

chromosomal localization of rat PC7, a novel mammalian proprotein convertase closest to yeast kexin-like proteinases. *Proc Natl Acad Sci U S A* **93,** 3388-3393.

Seidah, N. G., Marcinkiewicz, M., Benjannet, S., Gaspar, L., Beaubien, G., Mattei, M. G., Lazure, C., Mbikay, M., and Chretien, M. (1991a). Cloning and primary sequence of a mouse candidate prohormone convertase PC1 homologous to PC2, Furin, and Kex2: distinct chromosomal localization and messenger RNA distribution in brain and pituitary compared to PC2. *Mol Endocrinol* **5,** 111-122.

Seidah, N. G., Mattei, M. G., Gaspar, L., Benjannet, S., Mbikay, M., and Chretien, M. (1991b). Chromosomal assignments of the genes for neuroendocrine convertase PC1 (NEC1) to human 5q15-21, neuroendocrine convertase PC2 (NEC2) to human 20p11.1-11.2, and furin (mouse 7[D1-E2] region). *Genomics* **11,** 103-107.

Seidah, N. G., and Prat, A. (2002). Precursor convertases in the secretory pathway, cytosol and extracellular milieu. *Essays Biochem* **38,** 79-94.

Shennan, K. I., Taylor, N. A., Jermany, J. L., Matthews, G., and Docherty, K. (1995). Differences in pH optima and calcium requirements for maturation of the prohormone convertases PC2 and PC3 indicates different intracellular locations for these events. *J Biol Chem* **270,** 1402-1407.

Simons, K., and Toomre, D. (2000). Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1,** 31-39.

Smeekens, S. P., Avruch, A. S., LaMendola, J., Chan, S. J., and Steiner, D. F. (1991). Identification of a cDNA encoding a second putative prohormone convertase related to PC2 in AtT20 cells and islets of Langerhans. *Proc Natl Acad Sci U S A* **88,** 340-344.

Smeekens, S. P., Montag, A. G., Thomas, G., Albiges-Rizo, C., Carroll, R., Benig, M., Phillips, L. A., Martin, S., Ohagi, S., Gardner, P., and et al. (1992). Proinsulin processing by the subtilisin-related proprotein convertases furin, PC2, and PC3. *Proc Natl Acad Sci U S A* **89,** 8822-8826.

Smeekens, S. P., and Steiner, D. F. (1990). Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. *J Biol Chem* **265,** 2997-3000.

Smit, A. B., Spijker, S., Nagle, G. T., Knock, S. L., Kurosky, A., and Geraerts, W. P. (1994). Structural characterization of a Lymnaea putative endoprotease related to human furin. *FEBS Lett* **343,** 27-31.

Stawowy, P., Kallisch, H., Veinot, J. P., Kilimnik, A., Prichett, W., Goetze, S., Seidah, N. G., Chretien, M., Fleck, E., and Graf, K. (2004). Endoproteolytic activation of alpha(v) integrin by proprotein convertase PC5 is required for vascular smooth muscle cell adhesion to vitronectin and integrin-dependent signaling. *Circulation* **109,** 770-776.

Steiner, D. F. (1998). The proprotein convertases. *Curr Opin Chem Biol* **2,** 31-39.

Steiner, D. F., Cunningham, D., Spigelman, L., and Aten, B. (1967). Insulin biosynthesis: evidence for a precursor. *Science* **157,** 697-700.

Steiner, D. F., Patzelt, C., Chan, S. J., Quinn, P. S., Tager, H. S., Nielsen, D., Lernmark, A., Noyes, B. E., Agarwal, K. L., Gabbay, K. H., and Rubenstein, A. H. (1980a). Formation of biologically active peptides. *Proc R Soc Lond B Biol Sci* **210,** 45-59.

Steiner, D. F., Quinn, P. S., Chan, S. J., Marsh, J., and Tager, H. S. (1980b). Processing mechanisms in the biosynthesis of proteins. *Ann N Y Acad Sci* **343,** 1-16.

Stieneke-Grober, A., Vey, M., Angliker, H., Shaw, E., Thomas, G., Roberts, C., Klenk, H. D., and Garten, W. (1992). Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. *Embo J* **11,** 2407-2414.

Stock, S., and Osterlund, C. (1998). Expression of the oxytocin receptor and oxytocin gene in human oocytes and preimplantation embryos. *Adv Exp Med Biol* **449,** 323-324.

Tadros, H., Chretien, M., and Mbikay, M. (2001). The testicular germ-cell protease PC4 is also expressed in macrophage-like cells of the ovary. *J Reprod Immunol* **49,** 133-152.

Takahashi, S., Kasai, K., Hatsuzawa, K., Kitamura, N., Misumi, Y., Ikehara, Y., Murakami, K., and Nakayama, K. (1993). A mutation of furin causes the lack of precursor-processing activity in human colon carcinoma LoVo cells. *Biochem Biophys Res Commun* **195,** 1019-1026.

Takahashi, S., Nakagawa, T., Kasai, K., Banno, T., Duguay, S. J., Van de Ven, W. J., Murakami, K., and Nakayama, K. (1995). A second mutant allele of furin in the processing-incompetent cell line, LoVo. Evidence for involvement of the homo B domain in autocatalytic activation. *J Biol Chem* **270,** 26565-26569.

Tanaka, S., Kurabuchi, S., Mochida, H., Kato, T., Takahashi, S., Watanabe, T., and Nakayama, K. (1996). Immunocytochemical localization of prohormone convertases PC1/PC3 and PC2 in rat pancreatic islets. *Arch Histol Cytol* **59,** 261-271.

Taylor, N. A., Van De Ven, W. J., and Creemers, J. W. (2003). Curbing activation: proprotein convertases in homeostasis and pathology. *Faseb J* **17,** 1215-1227.

Thomas, G., Thorne, B. A., Thomas, L., Allen, R. G., Hruby, D. E., Fuller, R., and Thorner, J. (1988). Yeast KEX2 endopeptidase correctly cleaves a neuroendocrine prohormone in mammalian cells. *Science* **241,** 226-230.

Torii, S., Yamagishi, T., Murakami, K., and Nakayama, K. (1993). Localization of Kex2-like processing endoproteases, furin and PC4, within mouse testis by in situ hybridization. *FEBS Lett* **316,** 12-16.

Tsuji, A., Hine, C., Tamai, Y., Yonemoto, K., Mori, K., Yoshida, S., Bando, M., Sakai, E., Akamatsu, T., and Matsuda, Y. (1997). Genomic organization and alternative splicing of human PACE4 (SPC4), kexin-like processing endoprotease. *J Biochem (Tokyo)* **122,** 438-452.

Tsuji, A., Mori, K., Hine, C., Tamai, Y., Nagamune, H., and Matsuda, Y. (1994). The tissue distribution of mRNAs for the PACE4 isoforms, kexin-like processing protease: PACE4C and PACE4D mRNAs are major transcripts among PACE4 isoforms. *Biochem Biophys Res Commun* **202,** 1215-1221.

Uehara, M., Yaoi, Y., Suzuki, M., Takata, K., and Tanaka, S. (2001). Differential localization of prohormone convertases PC1 and PC2 in two distinct types of secretory granules in rat pituitary gonadotrophs. *Cell Tissue Res* **304,** 43-49.

Ugleholdt, R., Zhu, X., Deacon, C. F., Orskov, C., Steiner, D. F., and Holst, J. J. (2004). Impaired intestinal proglucagon processing in mice lacking prohormone convertase 1. *Endocrinology* **145,** 1349-1355.

van de Loo, J. W., Creemers, J. W., Bright, N. A., Young, B. D., Roebroek, A. J., and Van de Ven, W. J. (1997). Biosynthesis, distinct post-translational modifications, and functional characterization of lymphoma proprotein convertase. *J Biol Chem* **272,** 27116-27123.

van de Loo, J. W., Creemers, J. W., Kas, K., Roebroek, A. J., and Van de Ven, W. J. (1996). Assignment of the human proprotein convertase gene PCSK5 to chromosome 9q21.3. *Cytogenet Cell Genet* **75,** 227-229.

van de Loo, J. W., Teuchert, M., Pauli, I., Plets, E., Van de Ven, W. J., and Creemers, J. W. (2000). Dynamic palmitoylation of lymphoma proprotein convertase prolongs its half-life, but is not essential for trans-Golgi network localization. *Biochem J* **352 Pt 3,** 827-833.

Van de Ven, W. J., Roebroek, A. J., and Van Duijnhoven, H. L. (1993). Structure and function of eukaryotic proprotein processing enzymes of the subtilisin family of serine proteases. *Crit Rev Oncog* **4,** 115-136.

van de Ven, W. J., Voorberg, J., Fontijn, R., Pannekoek, H., van den Ouweland, A. M., van Duijnhoven, H. L., Roebroek, A. J., and Siezen, R. J. (1990). Furin is a subtilisin-like proprotein processing enzyme in higher eukaryotes. *Mol Biol Rep* **14,** 265-275.

Varlamov, O., Fricker, L. D., Furukawa, H., Steiner, D. F., Langley, S. H., and Leiter, E. H. (1997). Beta-cell lines derived from transgenic Cpe(fat)/Cpe(fat) mice are defective in carboxypeptidase E and proinsulin processing. *Endocrinology* **138,** 4883-4892.

Vey, M., Schafer, W., Reis, B., Ohuchi, R., Britt, W., Garten, W., Klenk, H. D., and Radsak, K. (1995). Proteolytic processing of human cytomegalovirus glycoprotein B (gpUL55) is mediated by the human endoprotease furin. *Virology* **206,** 746-749.

Vieau, D., Gangnon, F., Jegou, S., Danger, J. M., and Vaudry, H. (1998). Characterization of the cDNA encoding the prohormone convertase PC2 and

localization of the mRNA in the brain of the frog Rana ridibunda. *Brain Res Mol Brain Res* **63,** 1-13.

Villeneuve, P., Feliciangeli, S., Croissandeau, G., Seidah, N. G., Mbikay, M., Kitabgi, P., and Beaudet, A. (2002). Altered processing of the neurotensin/neuromedin N precursor in PC2 knock down mice: a biochemical and immunohistochemical study. *J Neurochem* **82,** 783-793.

Villeneuve, P., Seidah, N. G., and Beaudet, A. (1999). Immunohistochemical distribution of the prohormone convertase PC5-A in rat brain. *Neuroscience* **92,** 641-654.

Vishnuvardhan, D., Connolly, K., Cain, B., and Beinfeld, M. C. (2000). PC2 and 7B2 null mice demonstrate that PC2 is essential for normal pro-CCK processing. *Biochem Biophys Res Commun* **273,** 188-191.

Wang, J., Mayernik, L., and Armant, D. R. (2002). Integrin signaling regulates blastocyst adhesion to fibronectin at implantation: intracellular calcium transients and vesicle trafficking in primary trophoblast cells. *Dev Biol* **245,** 270-279.

Watson, A. J., Hogan, A., Hahnel, A., Wiemer, K. E., and Schultz, G. A. (1992). Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo. *Mol Reprod Dev* **31,** 87-95.

Westphal, C. H., Muller, L., Zhou, A., Zhu, X., Bonner-Weir, S., Schambelan, M., Steiner, D. F., Lindberg, I., and Leder, P. (1999). The neuroendocrine protein 7B2 is required for peptide hormone processing in vivo and provides a novel mechanism for pituitary Cushing's disease. *Cell* **96,** 689-700.

Wetsel, W. C., Liposits, Z., Seidah, N. G., and Collins, S. (1995). Expression of candidate pro-GnRH processing enzymes in rat hypothalamus and an immortalized hypothalamic neuronal cell line. *Neuroendocrinology* **62,** 166-177.

Winsky-Sommerer, R., Grouselle, D., Rougeot, C., Laurent, V., David, J. P., Delacourte, A., Dournaud, P., Seidah, N. G., Lindberg, I., Trottier, S., and Epelbaum, J. (2003). The proprotein convertase PC2 is involved in the maturation of prosomatostatin to somatostatin-14 but not in the somatostatin deficit in Alzheimer's disease. *Neuroscience* **122,** 437-447.

Wise, R. J., Barr, P. J., Wong, P. A., Kiefer, M. C., Brake, A. J., and Kaufman, R. J. (1990). Expression of a human proprotein processing enzyme: correct cleavage of the von Willebrand factor precursor at a paired basic amino acid site. *Proc Natl Acad Sci U S A* **87,** 9378-9382.

Yaoi, Y., Suzuki, M., Tomura, H., Kikuyama, S., and Tanaka, S. (2003). Molecular cloning and expression of prohormone convertases PC1 and PC2 in the pituitary gland of the bullfrog, Rana catesbeiana. *Zoolog Sci* **20,** 1139-1151.

Zheng, M., Seidah, N. G., and Pintar, J. E. (1997). The developmental expression in the rat CNS and peripheral tissues of proteases PC5 and PACE4 mRNAs: comparison with other proprotein processing enzymes. *Dev Biol* **181,** 268-283.

Zhong, M., Benjannet, S., Lazure, C., Munzer, S., and Seidah, N. G. (1996). Functional analysis of human PACE4-A and PACE4-C isoforms: identification of a new PACE4-CS isoform. *FEBS Lett* **396,** 31-36.

Zhong, M., Munzer, J. S., Basak, A., Benjannet, S., Mowla, S. J., Decroly, E., Chretien, M., and Seidah, N. G. (1999). The prosegments of furin and PC7 as potent

inhibitors of proprotein convertases. In vitro and ex vivo assessment of their efficacy and selectivity. *J Biol Chem* **274,** 33913-33920.

Zhou, A., Bloomquist, B. T., and Mains, R. E. (1993). The prohormone convertases PC1 and PC2 mediate distinct endoproteolytic cleavages in a strict temporal order during proopiomelanocortin biosynthetic processing. *J Biol Chem* **268,** 1763-1769.

Zhou, Y., and Lindberg, I. (1993). Purification and characterization of the prohormone convertase PC1(PC3). *J Biol Chem* **268,** 5615-5623.

Zhou, Y., and Lindberg, I. (1994). Enzymatic properties of carboxyl-terminally truncated prohormone convertase 1 (PC1/SPC3) and evidence for autocatalytic conversion. *J Biol Chem* **269,** 18408-18413.

Zhu, X., Orci, L., Carroll, R., Norrbom, C., Ravazzola, M., and Steiner, D. F. (2002a). Severe block in processing of proinsulin to insulin accompanied by elevation of des-64,65 proinsulin intermediates in islets of mice lacking prohormone convertase 1/3. *Proc Natl Acad Sci U S A* **99,** 10299-10304.

Zhu, X., Zhou, A., Dey, A., Norrbom, C., Carroll, R., Zhang, C., Laurent, V., Lindberg, I., Ugleholdt, R., Holst, J. J., and Steiner, D. F. (2002b). Disruption of PC1/3 expression in mice causes dwarfism and multiple neuroendocrine peptide processing defects. *Proc Natl Acad Sci U S A* **99,** 10293-10298.

APPENDIX 1. Table 5. Primers for RT-PCR

mRNA	Primer Sequences		Amplicon
	Strand[a]	Sequence (5'→3')	(bp)
PC1	S:	TGATTTTGCATGGGACATCTTCTC	396
	AS:	ACAGACTGTCTTCAGAGCCTTC	
PC2	S:	GAGACCCGTCTTCACGAATC	541
	AS:	GTTGAACCAGTCATCTGTGTATCG	
PC4	S:	CTGGGACAGATCTTCCCT	411
	AS:	GGTTCTCATCGTTGGGTGTGTA	
PC5	S:	GGGCGGAGAGGCCTTGGA	532
	AS:	TTTGTCGGTCTGTGCTTTCCAC	
PC7	S:	CCCACCCTGATGAGGAGAATG	434
	AS:	AAAGGCATCCGTCCCTCCTCA	
PACE4	S:	GCATAGAAAGGAATCACCCAG	462
	AS:	TGTAGCCATCACAGGAGCAG	
Furin	S:	TGAGCCATTCGTATGGCTACG	576
	AS:	GGACACAGCTTTTCTGGTGCA	
CPE	S:	CACGGAGGAGACCTTGTGGC	326
	AS:	TTGAGAGTCTCTTCAGGTGGG	
SAAS	S:	TTGGGCCTTCTGAGGCTGCC	498
	AS:	CACGTCAGGAGTCTCGTCGC	
7B2	S:	GTTTTGGTTGATCTTTGA	359
	AS:	CGTTTTCTAGACATCCATCA	
GAPDH	S:	TACGGCTACAGCAACAGGGT	235
	AS:	GGGTGCAGCGAACTTTATTG	

[a] S, sense; AS, antisense

APPENDIX 2. Table 6. Antibodies used in this study

Anti-	Type	Conjugated	Source	Host	Antigens	Dilution	
						IB	IIF
PC1-NT	Polyclonal	No	Dr. N.G. Seidah	Rabbit	$PC1^{112-128}$	1:4000	1:100
PC1-CT	Polyclonal	No	Dr. N.G. Seidah	Rabbit	$PC1^{629-726}$	1:4000	1:100
SAAS	Polyclonal	No	Dr. L. Fricker	Rabbit	$proSAAS^{245-260}$	N/A	1:500
CPE	Polyclonal	No	Dr. L. Fricker	Rabbit	$CPE^{426-433}$	1:2000	N/A
EGFP	Polyclonal	HRP	Clontech	Rabbit	GFP peptide	1:1000	1:100
rbIgG	Polyclonal	HRP	Amersham	Donkey	rabbit IgG	1:4000	N/A
mIgG	Polyclonal	HRP	Amersham	Sheep	mouse IgG	1:2000	N/A
rbIgG	Polyclonal	Alexa $Fluor^{594}$	Molecular Probes	Goat	rabbit IgG	N/A	1:200
mIgG	Polyclonal	Alexa $Fluor^{488}$	Molecular Probes	Goat	mouse IgG	N/A	1:200
Actin β	Monoclonal	No	Cedarlane	Mouse	chicken actin	1:250	N/A
Tubulin β	Monoclonal	No	Chemicon	Rat	Glu-Glu-Phe	N/A	1:250

CURRICULUM VITAE

Carly St. Germain

OBJECTIVE

Research Associate position with a biochemical research and development organization / basic research lab focusing on proteomics and molecular biology.

QUALIFICATIONS

Proteomics

- Practiced in both immunohistochemistry fluorescent microscopy and immunofluorescent confocal laser microscopy for protein visualization purposes.
- Skilled in cell culture work.
- Gained extensive experience in detecting protein via western blotting in both *in vivo* and *in vitro* systems.
- Experienced in protein profiling and data analysis using Ciphergen ProteinChip technology and software.

Molecular Biology

- Acquired skills in recombination, and purification of DNA as part of vector construction.
- Microbiology experience acquired through transformation and isolation of DNA in *E. Coli* bacterial system.
- Experience in DNA transfection into human cell lines (HEK293 and LoVo).

Reproduction

- Trained in a number of reproductive-related laboratory techniques such as mouse handling procedures, surgical oviduct removal and mouse embryo culture.

Other

- Advanced writing skills achieved through the preparation of manuscripts, abstracts, and theses.
- Excellent communication and presentation skills acquired through presentation of research and scientific journals as part of the Diseases of Ageing Seminar Series, weekly meetings and scientific conference attendance.

EDUCATION

- M.Sc. Biochemistry, University of Ottawa, 2002- 2004 (Supervisor, Dr. Majambu Mbikay)
  (Successful defence: October 19, 2004; Expected date of graduation: Spring Convocation, 2005).
- B.Sc. Honours Biochemistry, Bishop's University, 1999-2002.
  (Academic Honour Roll: 1999-2002)

LABORATORY RESEARCH HISTORY

- **M.Sc. research, Ottawa Health Research Institute (OHRI), Diseases of Ageing Group, University of Ottawa, 2002-2004**. Employed western blotting, immunofluorescent confocal laser microscopy, immunohistochemistry fluorescent microscopy, DNA, molecular biology, cell culture and mouse embryo culture techniques in determining the protein presence and localization of Proprotein Convertase 1 (PC1) in mouse preimplantation embryos and in attempting to explain its transient translocation to the nuclei of one cell fertilized embryos using an *in vitro* human cell line model.

- **Summer Student, Ottawa Health Research Institute (OHRI), Diseases of Ageing Group, 2002.** Gained experience on Ciphergen ProteinChip Array technology as a main user for the Diseases of Ageing Group

by conducting protein profiling and immunocapture assay experiments.

- **B.Sc. Honours Project, Université de Sherbrooke, 2002.** Construction of a fluorescent tagged vector: *PKDL-myc*. Gained experience in DNA, molecular biology, and cell culture techniques as well as fluorescent microscope operation.

POSTER/ PRESENTATION / PUBLICATIONS

Poster and Oral Presentations

- **C. St.Germain**, Gilles Croissandeau, J. Mayne, J. Baltz, M. Chrétien and M. Mbikay Expression and Transient Nuclear Translocation of Proprotein Convertase 1 (PC1) During Mouse Preimplantation Embryonic Development (2004). Canadian Workshop on Human Reproduction and Reproductive Biology (Ottawa, 2004), and Annual Ottawa Hospital Research Institute (OHRI) Research Day (University of Ottawa, 2004).

- ***C. St. Germain****, J. Mayne, M.C. Léveillé, and M. Mbikay. Biomarker Patterning During* In Vitro *Human Embryonic Development (2003). Annual OHRI Research Day (University of Ottawa, 2003) and Ottawa Reproductive Biology Workshop (Ottawa, 2003). Presented orally at Retreat for the Diseases of Ageing and Biochemical Neuroendocrinology Clinical Research Groups (Ottawa, 2002).*

Publications

- **C. St.Germain**, Gilles Croissandeau, J. Mayne, J. Baltz, M. Chrétien and M. Mbikay. Expression and Transient Nuclear Translocation of Proprotein Convertase 1 (PC1) During Mouse Preimplantation Embryonic Development (submitted 06/28/2004 to *Molecular Reproduction and Development*).

- ***C. St. Germain****, J. Mayne, M.C. Léveillé, B. Pak, A. Leader, M. Chrétien, J. Kelly, and M. Mbikay. Analysis of Spent Media from Human Embryos Culture by Surface-Enhanced Laser Desorption Ionization Time-of-Flight Mass Spectrometry (submission pending).*

COMPUTER SKILLS AND TRAINING

- **General**: Windows XP, Microsoft Office (Excel, PowerPoint, Word), WordPerfect, and Reference Manager.
- **Biological Science–Related**: Ciphergen ProteinChip Software, Confocal System BioRad (Ver. 3.2), Zeiss Axiovision 2.05, ImageJ 1.32j, PAWS, Gene Construction Kit 2.5, Ensembl, PUBMED
- **Training**: Workplace Hazardous Material Information System, Ottawa Hospital TOH Radiation Safety Training
- **Animal Handling**: Sacrifice of animal by cervical dislocation and surgical removal of mouse oviducts.

AWARDS RECEIVED

Biochemistry Departmental Entrance Scholarship from the University of Ottawa

- $4000/year (2002-2004)

H. Greville Smith Memorial Scholarship in Science from Bishop's University

- $2000/year (1999-2002)

WORK EXPERIENCE

- Gained experience in computer software testing and installation and receptionist duties through summer and part-time work with the federal public service at Industry Canada, Department of Public Works and Government Services, and Department of Indian Affairs and Northern Development (1997-2001).

LANGUAGES

- English
- French (Working Knowledge)

REFERENCES

- Available Upon Request