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GRADE / DEGREE

Biochemistry, Microbiology and Immunology
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

The Physiology and Biochemistry of the Fertility Enzyme Proprotein Convertase Subtilisin/Kexin Type 4

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ISBN: 978-0-494-59504-6
Our file Notre référence
ISBN: 978-0-494-59504-6

THE PHYSIOLOGY AND BIOCHEMISTRY OF THE FERTILITY ENZYME PROPROTEIN CONVERTASE SUBTILISIN/KEXIN TYPE 4

Charles Gyamera-Acheampong

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfilment of the requirements for the degree of

Doctor of Philosophy
in
Biochemistry

Department of Biochemistry, Microbiology, and Immunology
Faculty of Medicine
University of Ottawa

January 2009

DEDICATION

This thesis is dedicated to my deceased daughter, Harriet Ama Serwaa

ABSTRACT

This three-manuscript thesis focuses on the physiology and biochemistry of the fertility enzyme, Proprotein Convertase Subtilisin-Kexin type 4 (PCSK4). Efforts to develop effective, safe, and reversible methods of non-hormonal contraception especially for men have focussed either on sperm surface proteins involved in the functional maturation of sperm or on those responsible for sperm-egg interactions. PCSK4 is one of such proteins. It is a member of a family of serine endoproteinases involved in the proteolytic maturation of a wide array of inactive precursor proteins to their bioactive forms. It is primarily expressed in the gonads, and inactivation of its gene in mice causes male infertility and female subfertility.

The first paper dealt with the reproductive function of PCSK4 by studying its subcellular localisation in testicular epithelium and on intact sperm, as well as its relevance for sperm acquisition of fertilising ability. PCSK4 was detected in the acrosomal granules of round spermatids, in the acrosomal ridges of elongated spermatids, and on the sperm plasma membrane overlying the acrosome. Sperm from PCSK4-null mice underwent capacitation at a faster rate, they were induced to acrosome-react by lower concentrations of zona pellucida, and possessed egg-binding ability which was only half that of wildtype sperm.

The second paper explored the molecular basis of the ability of PCSK4-null sperm to undergo capacitation at a much faster rate than WT sperm, as well as their reduced egg-binding ability. It focussed primarily on sperm protein tyrosine phosphorylation and the proteolytic processing of the sperm-egg ligands ADAM2 and ADAM3. During sperm capacitation, proteins undergo more tyrosine phosphorylation and more ADAM2 proteolytic processing in PCSK4-null sperm than in WT sperm. Thus, alterations in signal transduction

and proteolytic processing during capacitation may underlie the fertilisation incompetence of PCSK4-null sperm.

The third paper investigated the biosynthesis, maturation, and transport of PCSK4. Mouse PCSK4 was identified to be tightly bound through hydrophobic interactions to water-insoluble or detergent-soluble components of the plasma membrane overlying the acrosome. Also human proPCSK4, cloned from human embryonic kidney cells (HEK293), is slightly converted into its active mature form, and it is probably retained inside the ER where it associates with Glucose-regulated protein 78/Immunogen Binding Protein (GRP78/BiP).

ACKNOWLEDGEMENTS

I would like to express my profound gratitude to my supervisor, Dr. Majambu Mbikay, for his immense contribution towards the completion of this thesis. He devoted his time and energy, as well as sought for financial support for almost the entire duration of the program. I am also grateful to the remaining members of my thesis advisory committee: Dr. Mary Alice Hefford and Dr. Fraser Scott, for their comments and suggestions which have improved the quality of this thesis. In particular, I am indebted to Dr. Mary Alice Hefford for the time she took, amidst a very tight schedule, in editing the second draft of the thesis. Worthy of appreciation are Drs. Gunther Schmidt, Ajoy Basak, Janice Mayne, Ms. Francine Sirois, Mrs. Adrianna Gambarotta, Mr. Andrew Chen, and Ms. Nathalie Scamuffa for their valuable contributions. I am very grateful to Natural Sciences and Engineering Research Council of Canada for providing the financial support needed to carry out this study.

I would like to extend my profound appreciation to my fellow graduate students in the Department of Biochemistry, Microbiology, and Immunology for the friendship and interactions we shared. I will particularly like to mention Hongbin Xu. My thanks further go to the support staff of BMI and the Chronic Disease Program of the Ottawa Hospital: Ms. Carol Ann Kelly, Ms. Nicole Trudel, Mrs. JoAnn McDonald, and Ms. Denise Joanisse for their extreme patience in dealing with all the needs and requests of graduate students.

I would never forget the support and prayers of Pastor Andrew Aryee, Church/family members, friends, and loved ones both far and near. May the Lord richly bless you all for the great interest you showed in my studies.

Last but not the least, to Mrs. Gyamera-Acheampong A., I say, a million thanks. As if having to support me spiritually, emotionally, and financially were not enough, you endured

the long hours I had to stay away from home so as to be in the laboratory. You might not be aware, but I want you to know that your love and encouragement contributed immensely to the successful completion of this thesis.

I cannot, within the page limitations, thank everyone that has made a contribution to this thesis, but I do acknowledge in my heart, all contributions, both great and small.

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LIST OF ABBREVIATIONS

ADAM	A Disintegrin and Metalloprotease
AEG	Acidic Epididymal Glycoprotein
AKAP	A Kinase Anchor Protein
ANOVA	Analysis of Variance
AR	Acrosome reaction
ASA	Antisperm Antibodies
ATP	Adenosine Triphosphate
BPN'	Bacterial Proteinase Novo
BSA	Bovine Serum Albumin
cAMP	Cyclic AMP
cDNA	Complementary DNA
CLGN	Calmegin
ChS	Cholesterol Sulphate
CMK	Chloromethyl ketone
CPD	Carboxypeptidase D
CPE	Carboxypeptidase E
CPTP	Chronic Postvasectomy Testicular Pain
CPIC	Complete Protease Inhibitor Cocktail
CRISP1	Cysteine Rich Secrotory Protein 1
CT	Carboxyl Terminal
CTC	Chlortetracycline
DAPI	4'-6-diamidino-2-phenylindole
DF	Decapacitation Factor
DlgA	Drosophila disc large tumour suppressor
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
DoP	Downstream of P Domain
DTT	Dithiothreitol
eCG	Pregnant mare's serum gonadotropin
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFP	Enhanced Green Fluorescent Protein
ELM	Eukaryotic Linear Motif
EPPIN	Epididymal Protease Inhibitor
EP140	Epididymal Protein 140
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated kinase

FA	Fertilisation Antigen
FITC	Fluorescein isothiocyanate
FPP	Fertilisation Promoting Peptide
FS	Fibrous Sheath
GAPDS	Glyceraldehyde-3-phosphate dehydrogenase-S
GPI	Glycosyl Phosphatidyl Inositol
gp20	Sialylglycoprotein 20
GRP78/BiP	Glucose Regulated Protein 78/Immunogen Binding Protein
HCG	Human chorionic gonadotropin
HCl	Hydrochloric Acid
HEK	Human Embryonic Kidney
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase
H6	Hexahistidinyl
IB	Immunoblotting
Ig	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IGF	Insulin-Like Growth Factor
IUPAC	International Union of Pure and Applied Chemistry
IP	Immunoprecipitation
IPTG	Isopropyl-β-D-thiogalactopyranoside
IVF	In vitro fertilisation
KO	Knockout
KRB	Krebs Ringer Bicarbonate
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LDH-C_4	Lactate dehydrogenase C_4
MAPK	Mitogen-Activated Protein Kinase
MBCD	Methyl Beta Cyclodextrin
MCS	Multiple Cloning Site
MFI	Male-factor infertility
mRNA	Messenger RNA
NARC-1	Neural Apoptosis Regulated Convertase-1
*N*B-DNJ	*N*-butyldeoxynojirimycin
NHS	Normal Horse Serum
NP-40	Nonyl phenoxylpolyethoxylethanol 40
NRDB	Non-reducdant database
NT	Amino Terminal
NZ	Naz and Zhu
OC	Oral Contraceptive

OCP	Oral Contraceptive Pill
OD	Optical Density
ODF	Outer Dense Fibre
Oligo	Oligodeoxynucleotide
ORF	Open Reading Frame
PACAP	Pituitary Adenylate Convertase Activating polypeptide
PACAP-LI	PACAP-like immunoreactivity
PACE	Paired Amino Acid Converting Enzyme
PBP1	Phosphatidylethanolamine binding protein 1
PBS	Phosphate Buffer Saline
PC	Proprotein Convertase
PCI	Protein C Inhibitor
PCR	Polymerase Chain Reaction
PCSK	Proprotein Convertase Subtilisin/Kexin-Like
P-DOMAIN	Protease Domain
PDI	Protein disulfide isomerases
PDILT	PDI-like of Testis
PDZ	PSD-95, DlgA, ZO-1
PH-20	Phosphatidylinositol-linked Hyaluronidase 20
PKA	Protein Kinase A
Prot. DE	Proteins D and E
PSD95	Postsynaptic Density Protein 95
PVDF	Polyvinylidene Fluoride
PVP	Post-Vasectomy Pain
PVPS	Post-Vasectomy Pain Syndrome
P1	Position 1
RGD	Arginine-Glycine-Aspartate
rhEppin	Recombinant human Eppin
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic Acid
RSA	Rabbit Serum autoantigens
RT-PCR	Reverse trancriptase Polymerase Chain Reaction
SC	Subcutaneous
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SIAA	Sperm Intra Acrosomal Antigen
SKI-1/SIP	Subtilin-Kexin Isozyme-1/Site 1 Protease
SOB2	Sperm Oocyte Binding Antigen 2
SP	Signal Peptide
SP-10	Sperm Protein 10
TCP-11	t-Complex 11
TEM	Transmission Electron Microscopy
TGC	Testicular Germ Cell

TM	Transmembrane Domain
tMDC	Testicular Metalloprotease-like/Disintegrin-like/Cysteine-rich Protein
Tris-HCl	2-Amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride
TX-100	Triton X-100
TX-114	Triton X-114
UN	United Nations
VIP	Vasoactive Intestinal Polypeptide
V5	Simian Virus 5
WHO	World Health Organisation
WT	Wildtype
ZO-1	Zonula Occludens-1 protein
ZP	Zona pellucida

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I. INTRODUCTION

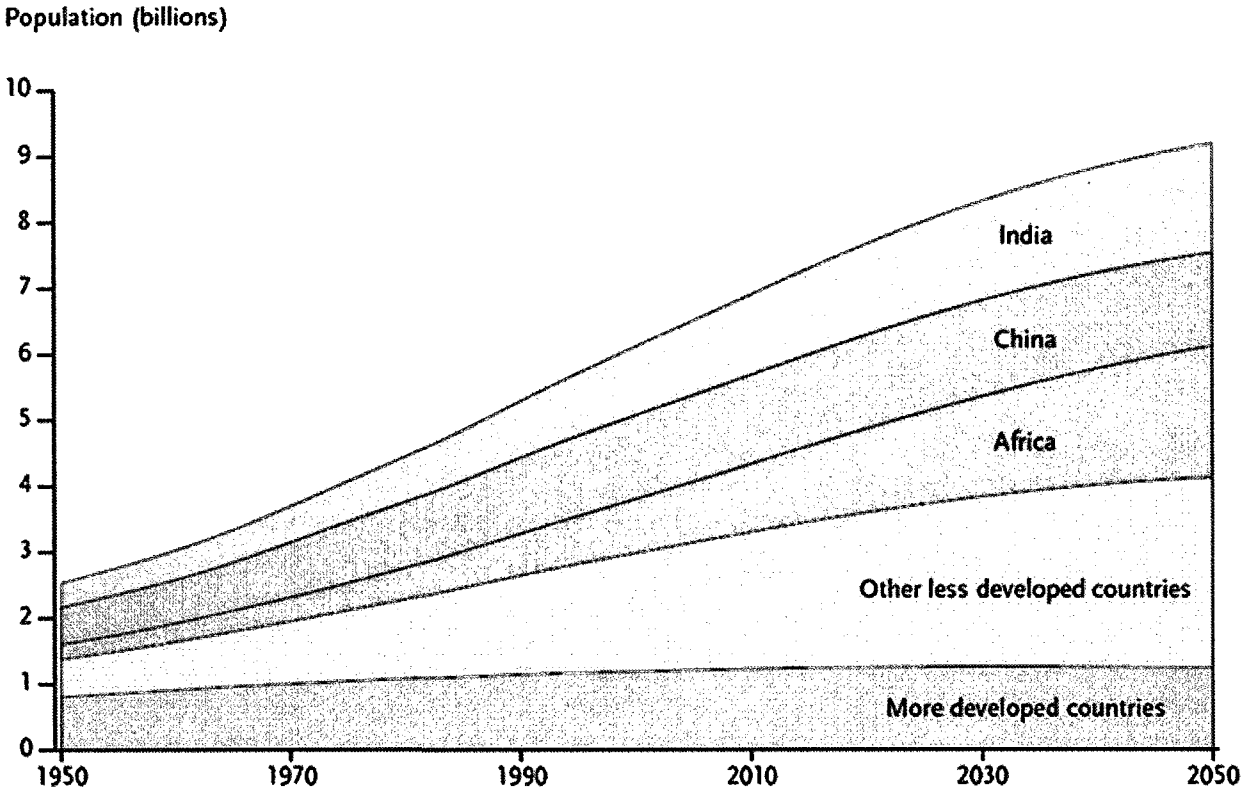
Proprotein Convertase Subtilisin/Kexin Type 4 (PCSK4) is an endoproteinase predominantly expressed in testicular germ cells and found on the plasma membrane overlying sperm acrosome. Genetic disruption of the *Pcsk4* gene in mouse leads to male infertility with no obvious developmental or hormonal abnormalities. This enzyme represents a plausible target in contraception. Its inhibition is likely to become an important addition to the panoply of approaches aimed at controlling birth rate and population growth.

This introduction reviews the current status of world population growth, the various methods now in use to control fertility, the alternative approaches under experimental exploration, as well as the biochemical properties of PCSK4 and its relevance to mammalian fertility.

I.1. NEED TO CONTROL WORLD POPULATION

The population of the world reached 1 billion in 1850, 2 billion in 1930, 2.5 billion in 1950, 3 billion in 1961, and 4 billion in 1976 (Kerr, 1995). It then rose from 5 billion in 1987 to 6.7 billion in 2008, and it is projected to hit 9.2 or 10 billion by 2050 (Kerr, 1995; Diekman and Herr, 1997; UN Population Division, 2007a; UN Population Division, 2007b). This presents a real risk of overpopulation with grave implications for the future. Within half a century, the proportion living in Africa, Asia, Latin America, and the Caribbean expanded from 68% to 80%; India and China, with more than a billion each in 2008 make up about 37% of the total; this trend is expected to continue (Fig. 1) (Population Reference Bureau, 2008).

Figure 1. *The world population*. Africa and other developing regions of the world make up an increasing share of the world population, and it is projected to hit about 9.2 or 10 billion by 2050. Adapted from (Population Reference Bureau, 2008).


Population (billions)
10
9
8
7
6
5
4
3
2
1
0
1950
1970
1990
2010
2030
2050
India
China
Africa
Other less developed countries
More developed countries

In contrast, the population in developed countries has remained relatively stable, and its proportion is even projected to drop from about 18% in 2008 to less than 14% by 2050 (Population Reference Bureau, 2008). It is interesting to note that, Africa's population, which was only 9% of the world's population in 1950, is currently growing faster than that of any other continent, and it is expected to account for about 21% of the world's population by 2050 (Population Reference Bureau, 2008).

Prior to the 1950s, mortality was the determining factor in population growth, however advances in medicine and improved living standards have drastically reduced death rates and fertility has now taken over as the dominating factor in population growth (Bongaarts, 1994; Spira, 1994). In view of this, effective methods of contraception, preferably, reversible forms, are needed to diminish the rapid population explosions in India, China, Africa, and other less developed countries, while maintaining a stable population growth in more developed countries.

I.2. HORMONAL CONTRACEPTION

Over the past five decades, women have borne most of the contraceptive burden; they have relied heavily on hormonal contraception such as combination oral contraceptive pills (OCPs), progestin-only pills, medroxyprogesterone acetate injections, or subdermal levonorgestrel implants (Schrager, 2002). Unfortunately, these have concomitant side effects.

I.2.1. Mode of action and efficacy of hormonal contraceptives

Most combination OCPs contain ethinyl oestradiol and synthetic progestin such as norethindrone, levonorgestrel, and norgestrel (Schrager, 2002). They function by inhibiting ovulation in most women by inducing cervical mucus thickening which then impedes transport of sperm to the uterus; about 0.1% of women become pregnant within the first year of using OCPs (Schrager, 2002). Progestin-only pills (mini-pills) prevent ovulation in about 50% of women, but the primary mode of action is cervical mucus thickening; about 0.5% of women become pregnant during the first year of use (Schrager, 2002). Contraceptive injections consisting of depot medroxyprogesterone acetate is an intramuscular 150 mg progestin injection that provides about 14 weeks of protection. The dose is quite high, ovulation is inhibited in most women, and about 0.3% of women become pregnant during the first year of use (Schrager, 2002). Contraceptive Implants consists of six subdermal implants that release constant low levels (0.05 to 0.08 mg per day) of the progestin, lenonorgestrel, over a five-year period. Ovulation is inhibited in most women; in addition, it induces thickening of the cervical mucus as well changes in the endometrium which block implantation; about 0.09% of women become pregnant during first year of use (Schrager, 2002).

I.2.2. Side effects of hormonal contraception

There are about 90 million females worldwide who use oral contraceptives (OCs) (Weber and Dohle, 2003). Though OCs are deemed reliable as measured by low Pearl Index (number of unwanted pregnancies per 100 women-years of use), half of every one million conceptions occurring every day worldwide are unintended (Henshaw, 1998; Weber and

Dohle, 2003). Moreover, as already mentioned, OCs have disturbing side effects despite their reported protection against epithelial ovarian cancer (The Cancer and Steriod Study Group, 1987; Parazzini *et al.*, 1991; Stanford, 1991; Whittemore *et al.*, 1992; Purdie *et al.*, 1995; Weiss and Rossing, 2001). It is interesting to note that, OCs usage and the reported benefit of ovarian cancer risk reduction has been challenged (Ness *et al.*, 2001).

The most frequent major adverse side effect of hormonal contraception is increased risk of cardiovascular diseases (Crook *et al.*, 1988; Godsland *et al.*, 1990; Farley *et al.*, 1998) such as thromboembolism which leads to stroke in women who smoke (Dugdale and Masi, 1971; Farley *et al.*, 1998), and increased blood pressure (Ribstein *et al.*, 1999; Curtis *et al.*, 2002; Lubianca *et al.*, 2003; Atthobari *et al.*, 2007).

Bleeding occurring at unpredictable times during the menstrual cycle, generally termed abnormal uterine bleeding (intermenstrual bleeding), is also a common problem associated with all forms of hormonal contraception (Schrager, 2002; Alders, 2004). Abnormal uterine bleeding may consist of breakthrough bleeding or spotting (Thorneycroft, 1999). Though abnormal uterine bleeding is rarely dangerous, many women find it worrisome. In fact, most women frequently stop using hormonal contraception due to irregular uterine bleedings (Rosenberg *et al.*, 1995; Schrager, 2002).

Other serious side effects include cervical cancer (Moreno *et al.*, 2002; Smith *et al.*, 2003), weight gain (Risser *et al.*, 1999), imbalances in cholesterol and lipid levels (Mishell *et al.*, 1997; Farley *et al.*, 1998), and interference with the female's natural hormonal system (Carol *et al.*, 1981). If taken for long periods of time, OCs can affect fertility (Hassan and Killick, 2004). It can also take a long time for natural menstrual cycles to be re-established when a user stops using OCs (Gnoth *et al.*, 2002).

Additional side effects such as nausea, bloating, irritability, or less sexual desire are also very common. These nagging problems associated with OCs result in their misuse or discontinuation, hence higher unintended pregnancies and increased abortion rates. In the United States alone, an estimated one third of the 3 million unintended pregnancies occurring each year are attributed to misuse or discontinuation of OCs (Weber and Dohle, 2003).

I.3. NON-HORMONAL CONTRACEPTION

I.3.1. Utilised by women

Contraception, in general, serves to prevent sexual intercourse from resulting in pregnancy. Non-hormonal contraception used by women works by either preventing sperm from fertilising an egg, or preventing the implantation of a zygote into the endometrium. The available techniques are vaginal rings (Mulders et al., 2002), intra uterine contraceptive devices, natural family planning (lactational, body temperature, rhythm, cervical mucus, etc.), spermicides, diaphragm, cervical caps, sponge, tubal sterilisation (Frank, 1999).

I.3.2. Utilised by men

About 45 million men worldwide have undergone vasectomy, 49 million use condoms, and 41 million rely on withdrawal (Martin *et al.*, 2000).

I.3.2.1. Vasectomy

Though vasectomy has been reported to be a highly effective method of non-hormonal contraception (Schwingl and Guess, 2000), it has some side effects which include haematoma (collection of blood outside the blood vessels), infection, sperm granuloma (accumulation of sperm in the obstructed duct allowing the infiltration of intraluminal macrophages) (McDonald, 2000), neuroma (inflammation and swelling of the surrounding nerves), epididymitis-orchitis (inflammation of one or both testicles), congestive epididymitis (PVPS: Post-vasectomy Pain Syndrome, PVP: Post-Vasectomy Pain, CPTP: Chronic Post-Vacsectomy Testicular Pain) which is chronic pain in the epididymides (McMahon *et al.*, 1992; Choe and Kirkemo, 1996; Ahmed *et al.*, 1997), and possible cardiovascular effects (Weber and Dohle, 2003). Virtually, all vasectomised men form antisperm antibodies (ASA) (Naz *et al.*, 1995; Diekman and Herr, 1997). Animals vasectomised for 2 or more years, have as much as four times, in diameter, enlarged ductuli as well as thickened epithelial basal lamina. Sperm become agglutinated in the lumen of the ductuli where they are ingested by macrophages (Alexander, 1972). As infiltrating macrophages in sperm granuloma phagocytise accumulated sperm, degradation products are absorbed via the epididymal epithelium; immune system is activated leading to ASA production (McDonald, 2000). There have however been no reported physiological complications despite the persistence of these antibodies for years after vasectomy (Naz *et al.*, 1995; Diekman and Herr, 1997).

I.3.2.2. Condoms and withdrawals

Condoms are highly effective against bacterial, viral, and parasite transmitted diseases, but a Pearl Index of 12 is quite high. Coitus interruptus or withdrawal, aside being highly inconvenient to men, has such a high pregnancy rate that Pearl Index is not used to measue it. The rate is 18 out of 100 (Weber and Dohle, 2003).

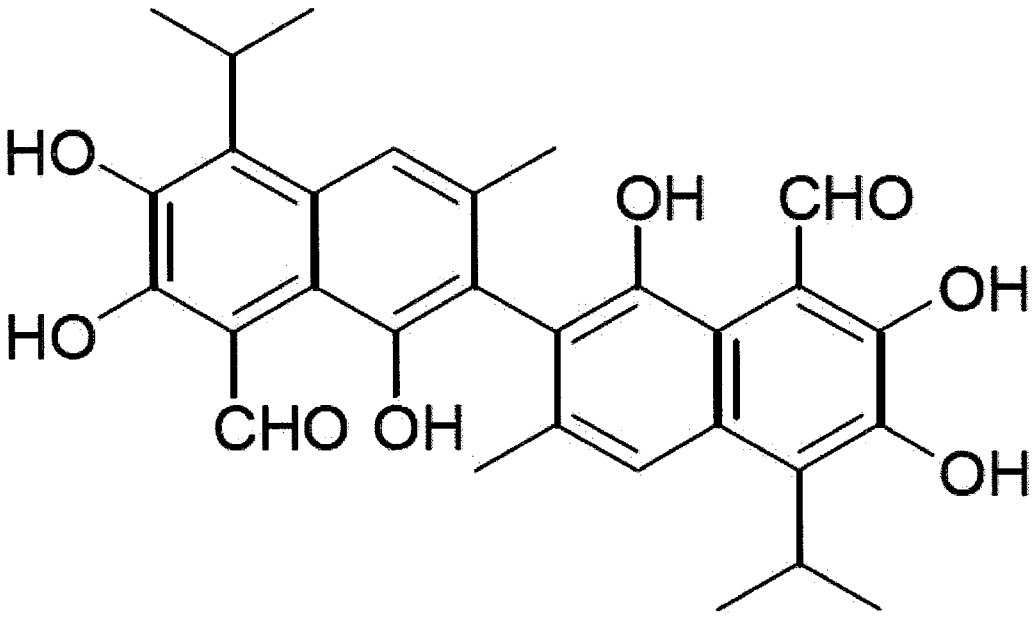
I.3.3. Experimental chemical contraceptives

Statistics show that men in many societies worldwide are now opening up to sharing the contraceptive responsibility with their female partners (Martin *et al.*, 2000), but many are not willing to accept current methods of injections, depot formulations, and various transdermal delivery systems (Weber and Dohle, 2003). The WHO task force has therefore identified as high priority, the development of new and effective methods of contraception, preferably, for men (Waites, 2003).

I.3.3.1. Gossypol

Gossypol (Fig. 2), a polyphenol compound present in the roots and stem of the cotton plant (*Gossypium sp.*), is very abundant in the seed oil as a yellow pigment. It is a natural defence in the plant by inducing infertility in insects that feed on its leaves and seeds. It permeates cells and inhibits several dehydrogenase enzymes such as LDH-C_4, and it has been shown to effectively suppress spermatogenesis in man (Weber and Dohle, 2003). The contraceptive potential of Gossypol was first discovered in China in the 1970s. The Chinese

Figure 2. *Gossypol*: Structure, molecular formula ($C_{30}H_{30}O_8$), and IUPAC name [2,2'-bis-(Formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene)].


HO
HO
CHO
OH
OH
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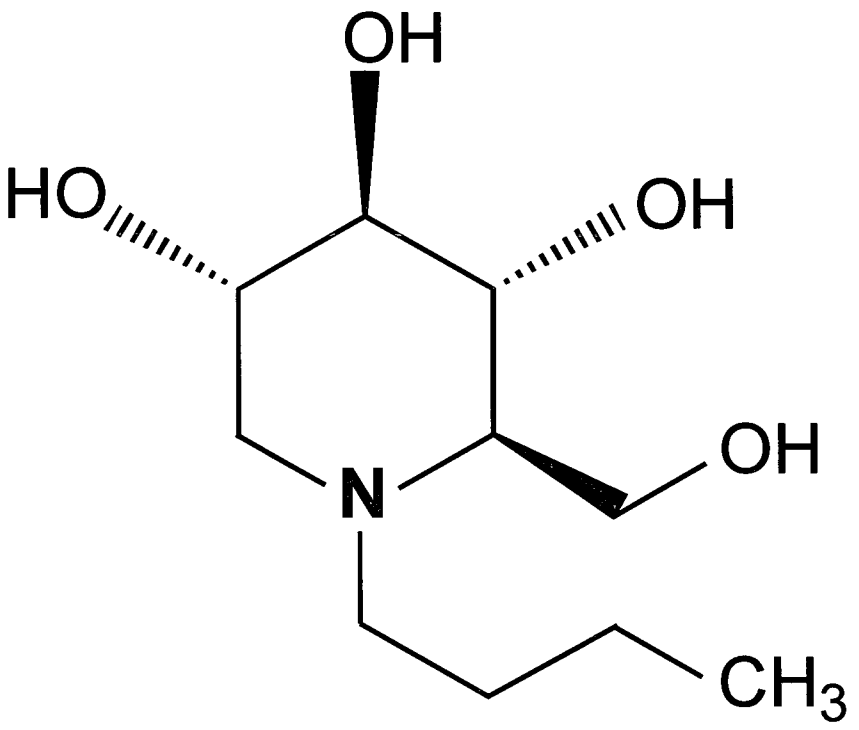
OH

Government, looking for ways of controlling the Chinese population, which was by then more than one billion, allowed clinical trials in about 8000 men; each man taking about 20 mg/day (Coutinho, 2002). Despite its tolerance and efficiency, it was not consistently reversible, it blocked calcium channels, and caused reduction in blood potassium levels (hypokalaemia) (Michael, 1998; Coutinho, 2002; Weber and Dohle, 2003). Owing to these side effects, scientists from China and the International Community with support from WHO declared that, the available forms of gossypol were too toxic to be developed for human contraception (Waites *et al.*, 1998; Coutinho, 2002; Weber and Dohle, 2003).

I.3.3.2. NB-DNJ

N-butyldeoxynojirimycin (*N*B-DNJ) (Fig. 3), an alkylated imino sugar, has been shown to cause reversible infertility in male mice (van der Spoel *et al.*, 2002). Glycolipids such as seminolipid (3-sulphogalactosyl-1-alkyl-2-acyl-*sn*-glycerol) and glycosphingolipids (complex ganglioside) are present in mammalian sperm (Ritter *et al.*, 1987; Ishizuka, 1997; Takamiya *et al.*, 1998), and spermatogenesis is severely impaired in seminolipid-null and complex ganglioside-null mice (Takamiya *et al.*, 1998; Fujimoto *et al.*, 2000; Honke *et al.*, 2002). The first step in the biosynthesis of gangliosides is the transfer of glucose to ceramide: a reaction catalysed by glucosyltransferase but inhibited by *N*B-DNJ (Platt *et al.*, 1994a; Platt *et al.*, 1994b; Dwek *et al.*, 2002). *N*B-DNJ also inhibits α-glucosidases I and II: two endoplasmic reticulum (ER) resident proteins involved in the processing of N-linked glycans present on glycoprotein (Elbein, 1987). The involvement of glycolipids in mammalian spermatogenesis led van der Spoel *et al.* to examine its effect(s) on fertilisation in mice and they reported its potential contraceptive properties (van der Spoel *et al.*, 2002).

Figure 3. *Alkylated Imino Sugar*: *N*-butyldeoxynojirimycin (*N*B-DNJ) $C_{10}H_{22}NO_4$.


OH
HO
OH
OH
N
CH3

Notwithstanding, it causes severe sperm head malformations, and the dosage required to block fertilisation, 15 – 150 mg/kg/day, is quite high.

I.3.4. Experimental immunocontraception

I.3.4.1. Anti-sperm antibodies

Attempts have also been made to look at Anti-Sperm antibodies (ASA) which have been reported to inhibit sperm function *in vitro*, induce infertility in experimental models, and implicated in some cases of infertility (Bronson *et al.*, 1984; Shulman, 1986; Diekman and Goldberg, 1994; Diekman and Herr, 1997). ASA are found in about 1% - 30% of infertile couples, and they have been reported to block fertilisation by decreasing acrosome reaction and/or zona pellucida binding, and postfertilisation effects (Peters and Coulam, 1992). Bivalent antibodies (antibody molecules with two or more binding sites that can crosslink one antigen particle to another) have the potential of agglutinating sperm resulting in the formation of immobile networks of cells (Diekman and Herr, 1997). When ASA binds to the sperm surface, they change the sperm swimming patterns, impair cervical mucus penetration, trap or agglutinate sperm in semen and cervical mucus, or invoke the complement cascade resulting in sperm lysis (D'Cruz *et al.*, 1991; Diekman and Herr, 1997; Frayne and Hall, 1999). There is also the possibility of Macrophage phagocytosis of the sperm in the female genital tract with the binding of ASA (Menge, 1971; Alexander, 1972; Marsh and Alexander, 1982; Peters and Coulam, 1992). ASA also have the potential of preventing sperm-egg/receptor-ligand interactions that control sperm binding to the zona

pellucida, zona penetration, and sperm-egg membrane adhesion/fusion (Diekman and Herr, 1997).

I.3.4.2. Search for contraceptive immunogens

Preferable contraceptive immunogens should therefore be sperm-specific to prevent cross-reactivities with immunogenic epitopes on somatic cell components, have a fertility-related function that can be inhibited by an antibody, be exposed on the surface of sperm to enable antibody recognition so as to affect sperm motility, be shown to inhibit fertility in animal models, and be able to inhibit pre-fertilisation events which is a preferred contraceptive method, instead of blocking implantation or inducing embryonic mortality which is unacceptable to many individuals (Herr, 1996; Frayne and Hall, 1999).

In 1932, Baskin reported the induction of temporary sterility in females by actively immunising them with human semen; apart from ethical issues, there was also the danger of anaphylactic and hypersensitivity reactions (Bernstein *et al.*, 1981; Kerr, 1995; Diekman and Herr, 1997). Aside the inherent dangers, injecting fresh semen into humans as a way of immunocontraception is ethically unacceptable. However, identifying and characterising suitable sperm antigens as effective and safe contraceptive immunogens are welcome.

Various immunocontraceptive candidate immunogens have been investigated (Table 1) (Anderson and Alexander, 1983; Hjort and Griffin, 1985; Frayne and Hall, 1999). On the egg, one antigen that has received considerable attention is ZP3, a major glycoprotein of the zona pellucida with sperm receptor function. Impaired fertility was observed in a number of female subjects of mammalian species (mouse, monkey, porcine, etc.) immunised with native ZP3, but there were frequent alterations in hormonal profiles and follicular

development (Gupta et al., 1997). Immunisation with synthetic peptides of ZP3 also resulted in the induction of autoimmune oophoritis and ovarian dysfunction (Rhim et al., 1992; Skinner et al., 1984). With respect to the sperm, immunisation of guinea-pigs with the sperm surface protein PH-20 leads to the induction of autoimmune orchitis (Tung et al., 1997).

Table 1. *Properties of sperm surface antigens implicated in sperm–egg interactions.*

a 'Seq' indicates whether the complete amino acid sequence of the sperm protein has been determined (normally deduced from the cloned cDNA sequence).

b 'Inhibition of binding in vitro' refers to the use of specific antisera, peptide mimics, or recombinant proteins in sperm–egg binding studies. 'Inhibition of binding in vivo' refers to immunisation trials in whole animals.

c Cyritestin has been alternatively localised to the inner acrosomal membrane (Linder *et al.*, 1995) or the equatorial segment (Yuan *et al.*, 1997) in two independent studies.

d The RSA (rabbit sperm autoantigen) family consists of three antigens; RSA 1, RSA 2 and RSA 3. Inhibition of binding studies, both in vitro and in vivo, have used a mixture of all three antigens. Additional data are available on RSA 3 alone (also known as SP17).

e Protein DE (also known as AEG), gp20 and EP140 are all epididymal secretory proteins which associate with the sperm surface during epididymal transit.

f The observed reduction in fertility in vivo was dependent on the strain of mouse. Adapted from (Frayne and Hall, 1999).

Table 1

Protein	Species	Seq[a]	Final location of mature protein	Binds to	inhibition of binding[b]	
					In vitro	In vivo
Fertilin β	Guinea-pig	Yes	Posterior head	Oolemma plasma membrane	Yes	Poor
	Rat	Yes	Acrosomal membrane			
	Mouse	Yes	Equatorial region & inner acrosomal membrane			
	Rabbit	Yes	Head			
	Bovine	Yes	Posterior head			
	Macaque	Yes	n.d.			
	Human	Yes	n.d.			
Fertilin α	Guinea-pig	Yes	n.d.	Oolemma plasma membrane	Yes	n.d.
	Rat	Yes	n.d.			
	Mouse	Yes	n.d.			
	Rabbit	Yes	Whole head			
	Macaque	Yes	n.d.			
	Human	Yes	Non-functional			
tMDC I/cyritestin	Mouse	Yes	Inner acrosomal membrane/equatorial seqment[c]	Oolemma plasma membrane	Yes	n.d.
	Rat	Yes	n.d.			
	Macaque	Yes	n.d.			
	Human	Yes	Non-functional gene			
PH-20 2B1	Guinea-pig	Yes	Plasma & inner acrosomal membrane of head	Penetration of cumulus via hyaluronidase activity: 2° zona binding	Yes	Yes
	Rat	Yes	Acrosomal			
	Mouse	Yes				
	Rabbit	Yes				
	Macaque	Yes	Head, plasma- & inner acrosomal membrane			
	Human	Yes	Head, plasma- & inner acrosomal membrane			

Table 1 (Continued)

Protein	Species	Seq[a]	Final location of mature protein	Binds to	inhibition of binding[b]	
					In vitro	In vivo
SP10	Human	Yes	Equatorial & acrosomal membrane	2° zona binding	Yes	n.d.
	Mouse	Yes	Intra-acrosomal membrane			
	Baboon	Yes	Intra-acrosomal membrane			
	Rabbit	Yes	Head			
	Macaque	Yes	n.d.			
	Fox	Yes	n.d.			
	Bovine	No	OMC & outer acrosomal membrane			
	Porcine	No	n.d.			
RSA family[d]	Rabbit	No	n.d.		Yes	Yes
SP17 RSA 3[C]	Mouse	Yes	Equatorial segment, principal & mid-piece	ZP3 carbohydrate moieties		Yes[f]
	Macaque	Yes	Plasma membrane principal piece			
	Human	Yes				
	Baboon	Yes				
	Rabbit	Yes		(R45 & R55 in rabbit)		
FA-1	Human	Part	Post acrosomal plasma membrane		Yes	Yes
	Mouse	Yes	n.d.			
	Rabbit	No	n.d.			
	Bovine	No	Post acrosomal	ZP3		
	Macaque	No	n.d.			
P26H	Hamster	No	Plasma membrane & acrosome	Zona	Yes	Yes
NZ-1	Mouse	Yes	n.d.	Zona		
SIAA	Human	No	Intra-acrosomal membrane	n.d.	Yes	n.d.

Table 1 (Continued)

Protein	Species	Seq[a]	Final location of mature protein	Binds to	inhibition of binding[b]	
					In vitro	In vivo
Zonadhesin	Mouse	Yes	n.d.	zona	n.d.	n.d.
	Porcine	Yes	n.d.			
Equatorin	Mouse	No	Equatorial	Oolemma plasma membrane	Yes	n.d.
	Rat	No	Equatorial			
	Human	No	Equatorial			
SOB2	Human	No	Post-acrosomal & neck	Oolemma plasma membrane	Yes	n.d.
Prot. DE/AEG[e]	Rat	Yes	Dorsal head & equatorial segment	Oolemma plasma membrane	Yes	n.d.
gp20[e]	Human	No	Equatorial	Oolemma plasma membrane	Yes	n.d.
EP140[e]	Rabbit	Part	Acrosome & midpiece plasma membrane	n.d.	n.d.	n.d.
LDH-C4	Baboon	Yes	Sperm tail	Causes sperm agglutination	n.d.	Yes
	Fox	Yes				
	Mouse	Yes				

I.3.4.3. Eppin

Epididymal protease inhibitor (Eppin) is a testis/epididymis specific protein (Richardson *et al.*, 2001; Sivashanmugam *et al.*, 2003). The mammalian epididymis is a specialised microenvironment in which epididymal secretory proteins confer maturity (forward motility and fertilisation capacity) on immature sperm by producing structural and biochemical changes in the sperm (Bedford, 1967; Orgebin-Crist, 1967; Kirchhoff *et al.*, 1998). Among the secretory proteins that ensure sperm surface modifications and maturation are serine proteases (Phelps *et al.*, 1990; He *et al.*, 1995; Kirchhoff *et al.*, 1997; Dacheux *et al.*, 1998), and Eppin is one of the major group of epididymal protease inhibitors that ensure homeostasis in the male reproductive tract (Poirier and Nicholson, 1984; Kirchhoff *et al.*, 1991; Perry *et al.*, 1993; Lee and Wei, 1994).

In 2004, Eppin was reported to be a possible contraceptive immunogen when fertile male monkeys were rendered infertile after immunising them with recombinant human Eppin (rhEppin) (O'Rand M *et al.*, 2004). The monkeys became infertile when each was initially immunised intramuscularly with 100 μg rhEppin followed by same quantity boostings every 21 days for up to 691 days. Interestingly, the monkeys regained their fertility 450 days after the immunisations were stopped (O'Rand M *et al.*, 2004). Though this approach was proven to be successful, the latency period and the number of immunogen administrations required for effective immunisation make this approach inconvenient and costly.

I.3.4.4. Recent progress in immunocontraception research

Recent attempts have leaned towards developing reversible forms of non-hormonal

contraception for males. They have focussed on targetting sperm surface proteins involved in or responsible for the functional maturation of sperm or sperm-egg interaction (see Table 1) (Kerr, 1995; Herr, 1996; Naz, 1996), and one such protein is PCSK4.

I.3.4.2.1. Fertilin α (ADAM1)

The fusion of the sperm and egg (fertilisation) marks the beginning of a series of events that convert a single cell into a multicellular organism. Several components on the sperm surface play active roles in mediating the successful binding and fusion of the gametes; among these are fertilin α, fertilin β, and cyritestin, respectively known as ADAM1, ADAM2, and ADAM3. ADAM stands for "A Disintegrin and Metalloprotease". Proteins in this family are also called MDCs: Metalloprotease/Disintegrin/Cysteine-rich proteins. They are structurally related cell surface proteins proposed to have cell adhesion activity, protease activity, or both (Blobel, 1997). ADAMs are biosynthesised as multidomain type-1 transmembrane preproproteins made up of a signal peptide, a prodomain, a metalloproteinase domain, a disintegrin domain, a cysteine-rich domain, an epidermal growth factor (EGF)-like domain, a transmembrane domain, and a cytoplasmic tail (Wolfsberg and White, 1996; Seals and Courtneidge, 2003). These proproteins undergo successive proteolytic cleavages, removing the prodomain and the metalloproteinase domain, leaving the disintegrin domain N-terminally exposed (Seals and Courtneidge, 2003). The first member of this family of proteins is Fertilin α (ADAM1).

Fertilin is a heterodimeric protein complex present on the sperm surface consisting of ADAM1 and ADAM2 (Blobel *et al.*, 1992; Lum and Blobel, 1997; Waters and White, 1997). There are two forms of Fertilin α in mouse testis: ADAM1a and ADAM1b (Nishimura *et al.*,

2002); ADAM1a is found in the ER of testicular germ cells whereas ADAM1b is found on the sperm surface (Kim *et al.*, 2003). Though ADAM1a and ADAM2 localise together in the endoplasmic reticulum of testicular germ cells (Cho *et al.*, 2000; Ikawa *et al.*, 2001; Kim *et al.*, 2003; Nishimura *et al.*, 2004; Kim *et al.*, 2006), only the complex between ADAM1b and ADAM2 is found on cauda epididymal sperm surface as an ADAM1b/ADAM2 heterodimer (Kim *et al.*, 2003). ADAM1a-deficient male mice are infertile because sperm have a severely impaired ability to migrate from the uterus into the oviduct through the uterotubal junction (Nishimura *et al.*, 2004). It is speculated that ADAM1a/ADAM2 complex may be involved in the transport of sperm proteins, including ADAM3, from the ER of testicular germ cells onto the cell surface (Nishimura *et al.*, 2004).

I.3.4.2.2. Fertilin β (ADAM2)

Sperm from mice lacking ADAM2 are infertile. Their sperm is deficient in sperm-egg membrane adhesion, sperm-egg fusion, migration from the uterus into the oviduct, and binding to the egg zona pellucida (Cho *et al.*, 1998). ADAM2 therefore enables sperm to migrate from the uterus into the oviduct, bind to the egg's ZP, and attach to the egg's membrane (Cho *et al.*, 1998). Sperm lacking ADAM2 also lacks fertilin α (Cho *et al.*, 2000) and have low levels (~10%) of ADAM3 (Nishimura *et al.*, 2001; Nishimura *et al.*, 2004; Stein *et al.*, 2005).

I.3.4.2.3. Cyritestin (ADAM3)

Male mice lacking ADAM3 are infertile. ADAM3 is not required for migration into the oviduct but is required for the binding to the egg's ZP and plasma membrane (Shamsadin

et al., 1999; Nishimura *et al.*, 2001). A complex between ADAM2 and ADAM3 has recently been reported to be present on the surface of testicular germ cells and epididymal sperm (Nishimura *et al.*, 2007). ADAM2 maybe required for the stabilisation of ADAM3 in sperm and the ADAM2/ADAM3 complex may be required for sperm-egg binding.

I.3.4.2.4. Hyaluronidase (PH-20)

In acrosome-intact sperm, PH-20 localises exclusively to the posterior head region but after acrosome reaction, it is found on the inner acrosomal membrane (Kerr, 1995). It is a GPI anchored surface hyaluronidase which hydrolyses hyaluronic acid, a polymer consisting of repeating disaccharides units of D-glucuronic acid and *N*-acetyl-D-glucosamine, present in the cumulus mass (Lin *et al.*, 1994). PH-20 mutant male mice are fertile (Baba *et al.*, 2002), however *in vitro*, their sperm possess reduced ability to disperse cumulus cells, which results in delayed fertilisation (Baba *et al.*, 2002). Lin *et al.* were able to successfully block the crossing of cumulus mass by incubating acrosome intact sperm with anti-hyaluronidase antibody (Lin *et al.*, 1994). Active immunisation of male and female guinea-pigs with PH-20 completely blocked fertility, however monoclonal antibodies raised against PH-20 did not bind to sperm from mouse, rat, hamster, or human (Kerr, 1995).

I.3.4.2.5. Lactate dehydrogenase C4 (LDH-C_4)

Lactate dehydrogenase C4 (LDH-C_4) is a sperm-specific but species-crossreactive isozyme of LDH produced by germ cells (Naz, 1996). Active immunisation of various species of animals with LDH-C_4 led to about 50% reduction in fertility through sperm agglutination and postfertilisation embryonic mortality. Notwithstanding, LDH-C_4 is a poor

immunogen as active immunisation of mice with whole sperm does not produce antibodies to LDH-C_4 (Naz, 1996), and sera from only about 10% infertile patients have modest elevated levels of antibodies to LDH-C_4 (Shelton and Goldberg, 1985). Although immunisation with LDH-C_4 has caused only about 50% reduction rather than a complete block of fertility in all the species tested so far, its sperm specificity and extensive characterisation make it an interesting model antigen for various aspects of immunocontraception (Naz, 1996).

I.3.4.2.6. Sperm Antigen 10 (SP-10)

SP-10 is an intra-acrosomal protein isolated and characterised from human sperm. After acrosome reaction, SP-10 is found on the inner acrosomal membrane and equatorial segment (Kerr, 1995). Monoclonal antibody (MHS-10) against SP-10 causes agglutination of sperm, however it has not been determined whether or not the sera from infertile patients have antibodies to SP-10 (Naz, 1996). Active immunisation trials have been performed in female baboons using the human recombinant SP-10. These baboons developed antibodies that were reactive with the cognate antigen. However, in spite of the presence of high titres of anti-SP-10 antibodies, there was only a partial reduction in fertility in a few animals (Kerr, 1995; Herr, 1996; Naz, 1996).

I.3.4.2.7. Fertilisation Antigen-1 (FA-1)

Fertilisation Antigen-1 (FA-1) is a glycoprotein purified and characterised from murine and human sperm/testis which exists as both a dimer and a monomer (Kerr, 1995; Naz, 1996). Anti-FA-1 completely blocked IVF in mouse, rabbit, bull, rhesus monkey, and humans *in vitro* (Kerr, 1995; Naz, 1996). Active immunisation of female rabbits with

purified FA-1 caused a significant reduction (up to complete block) in fertility (Naz, 1987) with the antibodies affecting sperm-zona interaction (Kerr, 1995; Naz, 1996).

I.3.4.2.8. Rabbit Sperm Autoantigens family (RSA family)

In rabbits, RSA antigens seem to function as lectin-like molecules mediating the binding of the sperm to the zona pellucida (Naz, 1996). Monoclonal antibodies to RSA antigens cross-react with human sperm and inhibit human sperm penetration of zona-free hamster oocytes (O'Rand and Irons, 1984). Female mice immunised with a synthetic peptide designated P10G (PGGGTLPPSG) led to a reduction in fertility. However, antibody response varied considerably among mice and in the high titre group of mice, there was only a reduction rather than a complete block of fertility (O'Rand *et al.*, 1993; Naz, 1996).

I.4. SPERM FUNCTIONAL MATURATION AND INTERACTION WITH EGGS

I.4.1. Protein phosphorylation

Phosphorylation is a reversible posttranslational modification that regulates protein activity. A majority of proteins in mammalian cells are phosphorylated at one or more sites (Olsen *et al.*, 2006). Phosphorylation involves the transfer of a phosphoryl group from ATP to a specific amino acid residue, and it is carried out in both prokaryotes and eukaryotes by kinases and reversed by phosphatases (Cozzone, 1988; Stock *et al.*, 1989; Barford *et al.*, 1998; Chang and Stewart, 1998). Many enzymes and receptors in biological systems are turned "on" and "off" by phosphorylation and dephosphorylation. The addition of a phosphoryl group to a specific amino acid residue or its removal from the same residue

induces a conformational change in the structure of the affected protein causing it to become active or inactive. Phosphorylation usually occurs on serine, threonine, and tyrosine residues in eukaryotic proteins, but on histidine and aspartate residues in prokaryotes (Cozzone, 1988; Stock *et al.*, 1989).

Phosphorylation at tyrosine residues mediates a variety of cellular functions such as growth regulation, cell cycle control, cytoskeleton assembly, ionic current regulation, and receptor regulation (Visconti and Kopf, 1998). In fact, phosphorylation of sperm proteins at tyrosine residues is one of the molecular events which take place during capacitation, a process that confers on the sperm the ability to fertilise.

Sperm capacitation is a complex process that results in a PKA-dependent tyrosine phosphorylation of proteins (Visconti and Kopf, 1998).

I.4.2. Sperm capacitation

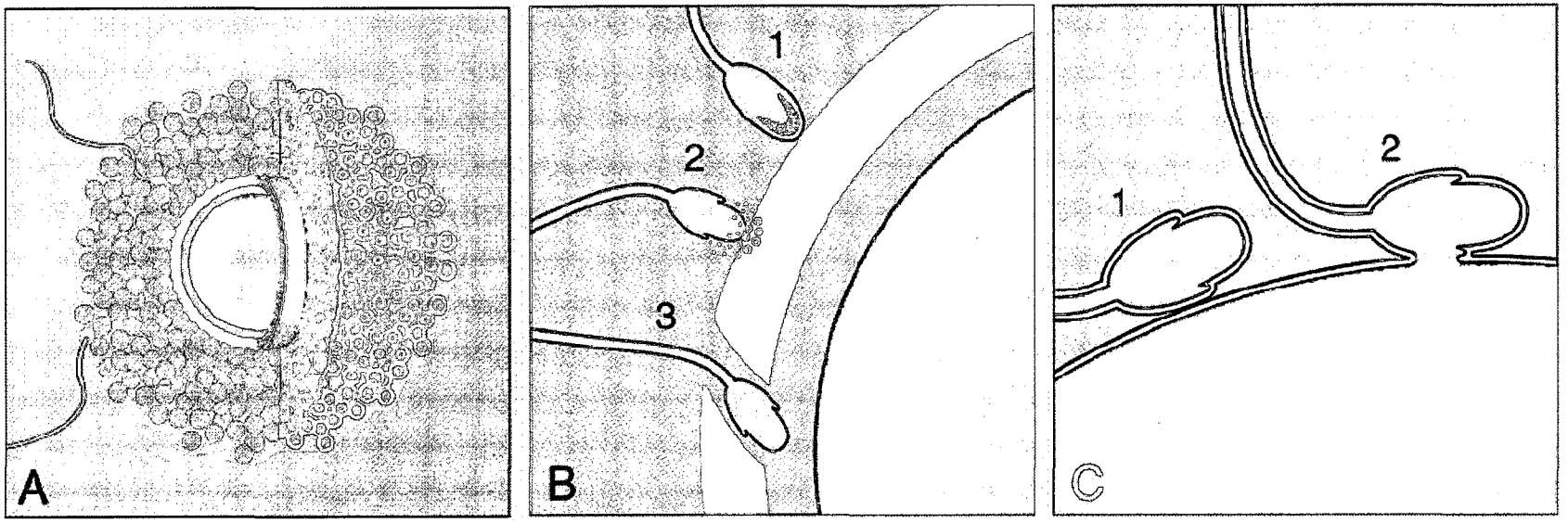
Though epididymal transit confers on sperm the ability to move progressively, when ejaculated, mammalian sperm are still fertilisation-incompetent; however, as they travel in the female genital tract towards the ovulated egg, they gain the ability to bind to eggs and fertilise (Chang, 1951; Austin, 1952). *In vitro* capacitation studies have unravelled some molecular events that take place during capacitation: among them, increased sperm metabolism (Hoppe, 1976; Fraser and Herod, 1990), changes in plasma membrane fluidity (Wolf *et al.*, 1986), changes in lectin reactivity (Johnson and Hunter, 1972; Talbot and Franklin, 1978; Talbot *et al.*, 2003), hyperactivated motility of sperm (Yanagimachi, 1994; Ho and Suarez, 2001), elevated intracellular pH (Zeng *et al.*, 1996), membrane hyperpolarisation (Demarco *et al.*, 2003), and increased protein tyrosine phosphorylation

(Visconti *et al.*, 1995). The physiological and biochemical changes which includes reorganisation of membrane proteins, metabolism of membrane phospholipids, reduction in membrane cholesterol levels, and hyperactivated motility (Yanagimachi, 1994); that confer on the sperm, the ability to fertilise, is termed Capacitation (Chang, 1951; Austin, 1952; Chang, 1955).

I.4.3. Sperm-egg interaction

Capacitation results in altered patterns of sperm motility (Primakoff and Myles, 2002), and sperm penetration of nearly 3000 cumulus cells surrounding an egg through the assistance from PH-20 (Fig. 4A) (Salustri *et al.*, 1992; Primakoff and Myles, 2002). Fertilisation is achieved when receptors on the sperm head surface bind to ligands on the egg's zona pellucida (ZP). The binding triggers sperm intracellular signalling cascades that stimulate acrosomal exocytosis, a process termed as acrosome reaction (Fig. 4B) (Yanagimachi, 1994; Baldi *et al.*, 2002; Primakoff and Myles, 2002; Visconti *et al.*, 2002). Hydrolysing enzymes within the acrosome perforate the ZP matrix layer, paving the way for the sperm to enter into the perivitelline space to initiate sperm-egg fusion (Fig. 4C) (Primakoff and Myles, 2002).

Figure 4. *Diagrammatic representation of mammalian sperm-egg interaction.* **A)** Sperm penetration of cumulus cells (purple) to reach zona (navy blue). **B)** Egg depicted with cumulus cells removed; sperm 1 binds to the zona pellucida (navy blue); sperm 2 undergoes exocytosis, releasing acrosomal contents (orange-red); sperm 3 penetrates the zona pellucida and begins entry into perivitelline space (gray). **C)** Sperm 1 binds to the egg plasma membrane by the side of its head, in a central region (equatorial region); sperm 2 fuses with the egg plasma membrane. Adapted from (Primakoff and Myles, 2002).


A
B
1
2
3
C
1
2

I.5. BIOCHEMISTRY AND BIOLOGY OF PCSK4

I.5.1. Limited endoproteolysis as a regulatory mechanism

Limited endoproteolysis is a post-translational modification by which cells diversify and regulate the products of their genes. In the secretory pathway, it is crucial for activation or inactivation of many proteins, as well as the regulation of their cellular localisation (Bergeron et al., 2000). In fact, endoproteolytic processing is crucial for the activation of many precursor proteins involved in such important biological functions as zymogen activation, peptide hormone processing, complement activation, clot formation/lysis, angiogenesis, and tissue remodelling (Steiner, 1998; Seidah and Prat, 2002). In the secretory pathway of eukaryotes, this processing occurs mostly at the carboxyl (C) side of Lys or Arg residues. An amino acid residue is said to be at P1 when located at the position directly preceding the scissile bond, at P2 when at the penultimate position, etc. It is said to be at the P1' when located directly after the scissile bond, at P2' when at the following position etc. (Schechter and Berger, 1967).

I.5.2. Proprotein convertases

I.5.2.1. The PCSK family

Limited endoproteolysis in the secretory pathway of mammalian cells is carried out, in large part, by the enzymes belonging to the Proprotein Convertase Subtilisin-Kexin (PCSK) family; a 9-member family of calcium-dependent serine endoproteinases structurally related to bacterial Subtilisin and yeast Kexin (Seidah *et al.*, 1999; Bergeron *et al.*, 2000).

Members of this family discovered to date are PCSK1, PCSK2, PCSK3, PCSK4, PCSK5, PCSK6, PCSK7, PCSK8, and PCSK9 otherwise known as PC1/3, PC2, furin, PC4, PC5/6, PACE4, PC7/8, subtilisin kexin isozyme-1/site 1 protease (SKI-1/SIP), and Neural Apoptosis Regulated Convertase-1 (NARC-1), respectively (Seidah and Chretien, 1999; Zhou *et al.*, 1999; Naureckiene *et al.*, 2003; Seidah *et al.*, 2003). These enzymes are expressed in nearly all nucleated cells in different combinations. PCSK1 and PCSK2 are mostly found in endocrine and neuroendocrine cells; PCSK3, PCSK5, PCSK6 and PCSK7 are widely expressed; PCSK4, mainly in the gonads (Seidah *et al.*, 1992; Lusson *et al.*, 1993; Nakagawa *et al.*, 1993; Seidah and Chretien, 1999; Zhou *et al.*, 1999). PCSK1-7 belong to the kexin subfamily; PCSK8 and PCSK9 belong to the pyrolysin and proteinase K subfamilies, respectively (Fig. 5) (Seidah *et al.*, 2006).

I.5.2.2. PCSK functional domains

PCSKs are biosynthesised as secretory precursor proteins made of several successive domains: a signal peptide (SP), a prodomain, a catalytic domain, a Protease (P) domain (except for PCSK9), and a carboxyl terminal (CT) domain (Fig. 6). The SP routes the nascent polypeptide into the secretory pathway. It is removed by a signal peptidase after the translocation of the zymogen through the ER membrane. The prodomain serves as an intramolecular chaperone ensuring proper folding of the proteins to which they are attached. The cleavage of the prodomain of PCSKs is autocatalytic (Leduc *et al.*, 1992; Creemers *et al.*, 1993; Goodman and Gorman, 1994; Matthews *et al.*, 1994; Lamango *et al.*, 1999), and it also ensures the correct sorting of the enzymes out of the ER (Creemers *et al.*, 1995; Zhou *et al.*, 1995). When the prodomain is cleaved, it still remains attached to the mature enzyme

Figure 5. *Classification of the proteases of the genome with emphasis on the PCSKs.* The five classes of proteases are represented on the left with the nucleophile used in each enzyme class. The emphasis is on serine proteases of the subtilisin type, of which six subfamilies within the clan SB can be identified. The basic amino acid PCSKs are related to yeast kexin, while the convertases PCSK8 and PCSK9 are related to pyrolysin and proteinase K, respectively. Adapted from (Seidah *et al.*, 2006).

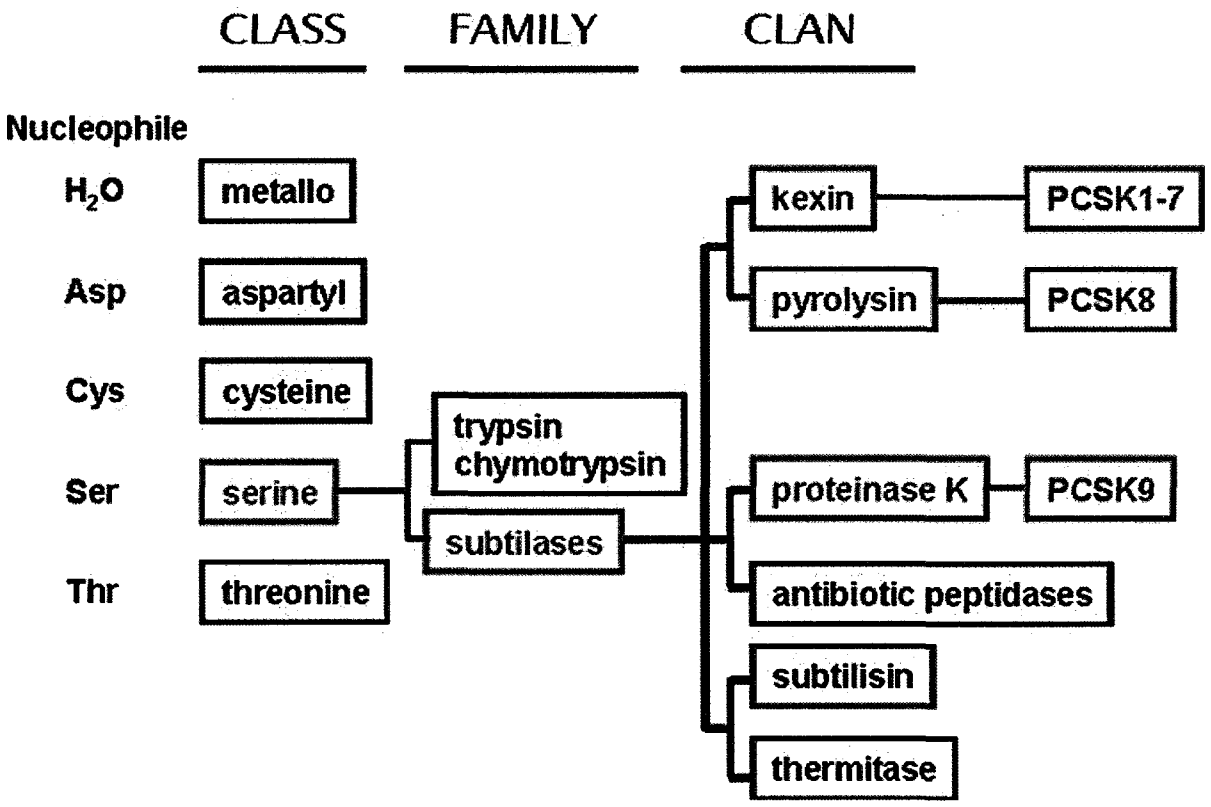
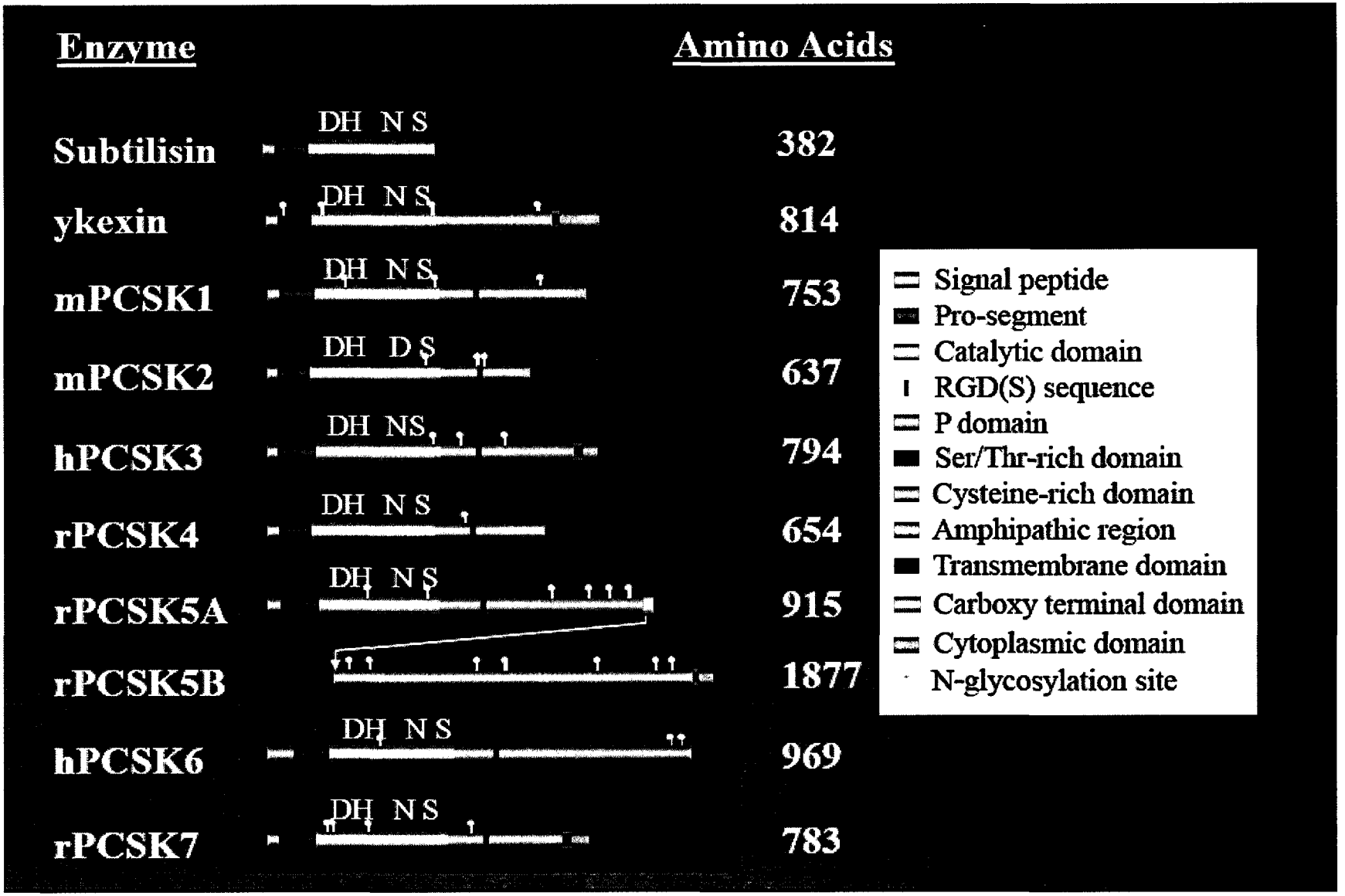

CLASS
FAMILY
CLAN
Nucleophile
H_2O
Asp
Cys
Ser
Thr
metallo
aspartyl
cysteine
serine
threonine
trypsin
chymotrypsin
subtilases
kexin
PCSK1-7
pyrolysin
PCSK8
proteinase K
PCSK9
antibiotic peptidases
subtilisin
thermitase

Figure 6. *The Proprotein convertase subtilisin/kexin-like family.* Diagrammatic representation of the structural motifs in mammalian proprotein convertases and their comparison to bacterial subtilisin BPN' and yeast kexin. Adapted from (Seidah and Chretien, 1999).


Enzyme
Amino Acids
Subtilisin
DH N S
382
ykexin
DH N S
814
mPCSK1
DH N S
753
mPCSK2
DH D S
637
hPCSK3
DH NS
794
rPCSK4
DH N S
654
rPCSK5A
DH N S
915
rPCSK5B
1877
hPCSK6
DH N S
969
rPCSK7
DH N S
783
Signal peptide
Pro-segment
Catalytic domain
RGD(S) sequence
P domain
Ser/Thr-rich domain
Cysteine-rich domain
Amphipathic region
Transmembrane domain
Carboxy terminal domain
Cytoplasmic domain
N-glycosylation site

thereby acting as a competitive inhibitor; its removal renders the mature enzyme fully active (Anderson *et al.*, 1997). The catalytic domain contains (i) the active site of the enzyme which consists of the His, Asp, Ser catalytic triad typical of serine proteases; (ii) a conserved Asn residue which ensures the stability of the oxyanion hole transiently formed during substrates hydrolyses; (iii) a P domain, which by virtue of its ability to influence the enzyme's dependence on calcium and pH, ensures correct folding and stability; (iv) an Arg-Gly-Asp (RGD) integrin-binding motif located within the P domain. The CT domain, the most variable among PCSK family members, mediates protein-protein interactions and sometimes carries a transmembrane (TM) domain (Seidah and Chretien, 1999; Bergeron *et al.*, 2000; Seidah and Prat, 2002; Seidah *et al.*, 2008).

I.5.2.3. PCSK cleavage specificities

Kexin-like convertases cleave their substrates on the carboxyl side of a P1 Arg, most often preceded by Lys or Arg at P2, P4, P6 or P8 positions. The general motif recognised is $(H/R/K)X_n(R/K)\downarrow$ where X_n is any amino acid except Cys or Pro; and n = 0, 2, 4, or 6 amino acids between the basic residues (Seidah and Chretien, 1999; Zhou *et al.*, 1999). After the cleavage of a precursor protein by a kexin-like convertase, the exposed basic residues of the released N-terminal peptide are removed by carboxypeptidase D (CPD) or carboxypeptidase E (CPE) (Kemmler *et al.*, 1973; Dong *et al.*, 1999; Bergeron *et al.*, 2000). If the action of a carboxypeptidase results in a peptide with a CT glycine, this residue is converted into an amide group by peptidyl-glycine-α-amidating mono-oxygenase. This conversion has been

shown to promote the binding of several neuroendocrine peptides to their receptor, as well as to increase their stabilities *in vivo* (Bergeron *et al.*, 2000).

PCSK8 cleaves its substrates after non-basic residues within the motifs (R/K)XX$^{(hydrophobic)}$Z↓ where Z is any amino acid, preferably Leu or Thr, but not Val, Pro, Glu, Asp, or Cys (Espenshade *et al.*, 1999; Seidah and Chretien, 1999; Elagoz *et al.*, 2002). PCSK9 cleaves itself at (V/I)FAQ↓ motif at end of the prodomain (Benjannet *et al.*, 2004). Collectively, PCSKs are responsible for the proteolytic activation of a variety of precursor proteins in the secretory pathway. Functional products of this modification include prohormones, proneuropeptides, growth factors, blood coagulation factors, plasma proteins, receptors, extracellular matrix (ECM) proteins, transcription factors, viral glycoproteins, and bacteria toxins (Seidah and Chretien, 1999; Zhou *et al.*, 1999).

I.5.3. PCSK4: A Gonadal Convertase

I.5.3.1. Structure, expression, and activity

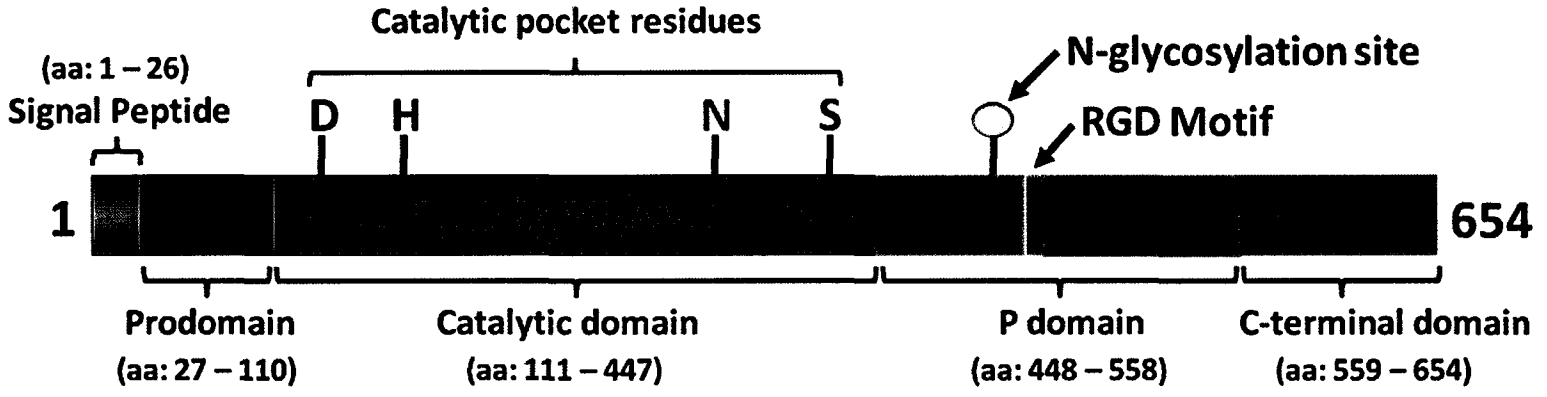
PCSK4 is specified by a 9.5-kb gene containing 15 exons and 14 introns (Mbikay *et al.*, 1994). The organisation of the exons and introns is similar to those for PCSK1, PCSK2, and PCSK3 (Seidah *et al.*, 1990; Smeekens and Steiner, 1990; van den Ouweland *et al.*, 1990; Seidah *et al.*, 1991). The gene maps to chromosome 10 in mouse (locus symbol, *Pcsk4*) and to chromosome 19 in human (locus symbol, *PCSK4*) (Mbikay *et al.*, 1995). In either mouse or rat, it is transcribed into a 2.8-kb mRNA and 5 alternatively spliced isoforms (Nakayama *et al.*, 1992; Seidah *et al.*, 1992; Mbikay *et al.*, 1994). Out of the 7 known kexin-like convertases, the distribution of PCSK4 is the most restricted, suggesting a unique

transcriptional regulation of the gene (Bergeron *et al.*, 2000). *Pcsk4* transcripts are detectable by *in situ* hybridisation near the lumen of rodent testicular tubules (Seidah *et al.*, 1992; Bergeron *et al.*, 2000), and by Northern blot analysis in testicular cell fractions enriched in spermatocytes and round spermatids (Seidah *et al.*, 1992; Torii *et al.*, 1993). Worthy of note is the fact that, *in situ* hybridisation analysis of adjacent sections of rat seminiferous tubules also reveals the expression of PCSK7 in a mutually exclusive pattern: some tubules contain greater levels of *Pcsk4* mRNA while others are rich in *Pcsk7*; suggesting distinct roles of both PCSKs in different stages of germ cell maturation (Bergeron *et al.*, 2000). *Pcsk4* transcripts, which are first observed in mouse testis on postnatal day 16 (p16) after primary spermatocytes have appeared (Bellvé, 1993; Tadros *et al.*, 2001), are absent in spermatogonia, elongated spermatids, Sertoli cells, or Leydig cells. In the ovary, the transcripts are found in very low amounts in macrophage-like cells (Tadros *et al.*, 2001).

The major *Pcsk4* mRNA encodes for a secretory precursor glycoprotein of 654 and 655 amino acids in rat and mouse, respectively (Nakayama *et al.*, 1992; Seidah and Prat, 2002). The protein is also found in the same germ cells (spermatocytes and round spermatids) as the transcripts, but persists and accumulates in elongated spermatids and in sperm. In the sperm, it is found on the plasma membrane overlying the acrosome (Gyamera-Acheampong *et al.*, 2006). In the *Xenopus*, PCSK4 expression is restricted to the ovaries and testes, however, low levels of the transcript are present in the brain (Nelsen *et al.*, 2005). PCSK4 has also been reported to be present in human placental cell line (Qiu *et al.*, 2005).

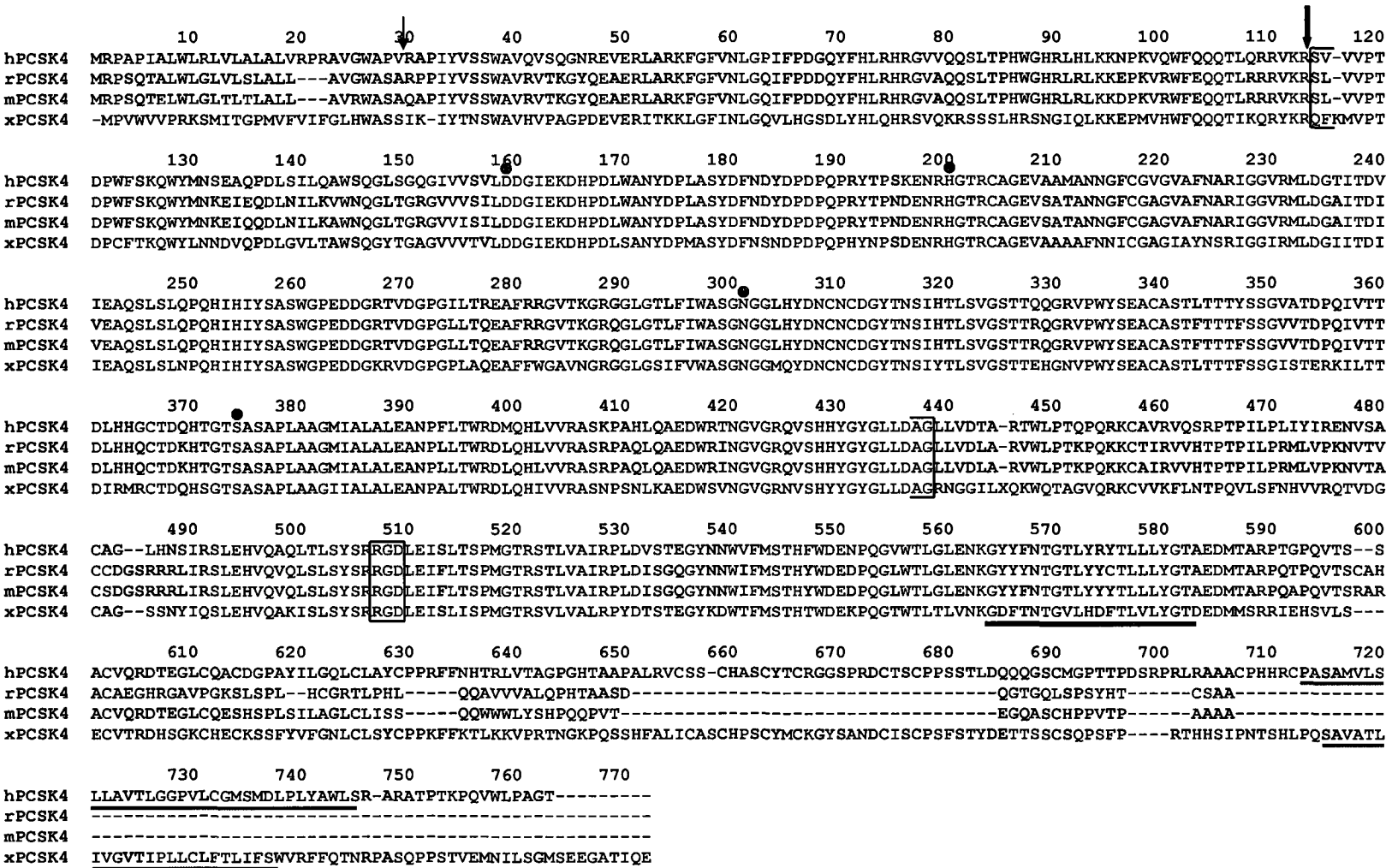
The encoded polypeptide in mouse or rat is made up of an N-terminal hydrophobic signal peptide, a prodomain, a subtilisin/kexin-like catalytic domain, a P domain, and a C-terminal domain (Fig. 7). Like other kexin-like PCSKs, PCSK4 cleaves its substrates at the carboxyl side of an Arg, when this P1 Arg is preceded by another basic amino acid at P2

Figure 7. *Diagrammatic representation of rPCSK4 domains.* PCSK4, like other PCSKs, possesses a signal peptide, a prodomain, a catalytic domain which contains the active site catalytic triad, a P domain (HomoB domain) containing a conserved RGD motif found in extracellular matrix proteins, and a Carboxyl-terminal domain, which is highly variable among the convertases (Nakayama *et al.*, 1992; Seidah *et al.*, 1992).


Catalytic pocket residues
(aa: 1 – 26)
Signal Peptide
D
H
N
S
N-glycosylation site
RGD Motif
1
654
Prodomain
(aa: 27 – 110)
Catalytic domain
(aa: 111 – 447)
P domain
(aa: 448 – 558)
C-terminal domain
(aa: 559 – 654)

(Lys-Arg↓; Arg-Arg↓) and/or at P4 (Lys-X-X-Arg↓; Arg-X-X-Arg↓, where X stands for any amino acid except Cys or Pro). Among the PCSKs, PCSK4 is uniquely efficient at cleaving at Lys-X-X-Arg↓ sites (Basak *et al.*, 1999; Basak *et al.*, 2004). Except for the C-terminal domain, the PCSK4 sequence is highly conserved between human, rat, mouse, and frog (Fig. 8). The sequence similarity between hPCSK4 and rPCSK4 is 67%; hPCSK4/mPCSK4 is 68%; hPCSK4/xPCSK4 is 56%; rPCSK4/mPCSK4 is 90%; rPCSK4/xPCSK4 is 49%; and mPCSK4/xPCSK4 is 49.5%.

Figure 8. *The sequence alignment of hPCSK4, rPCSK4, mPCSK4 and xPCSK4.* Conserved residues among the four species are given in red, those conserved between three of the four in blue, and those conserved between two in black (Sequence identities: hPCSK4 *vs* rPCSK4 = 67%; hPCSK4 *vs* mPCSK4 = 68%; hPCSK4 *vs* xPCSK4 = 56%; rPCSK4 *vs* mPCSK4 = 90%; rPCSK4 or mPCSK4 *vs* xPCSK4 = 49.4%). Gaps in the alignment are indicated by hyphens. The canonical residues of the catalytic pocket of serine proteases are highlighted with green dots. The cleavage sites for the removal of the signal peptide and the prodomain are indicated with thin arrow and thick arrows, respectively. The catalytic domain is delimited with brackets The RGD motif that may mediate interaction with integrins is boldly boxed. A CT hydrophobic motif is boldly underlined in black, and the transmembrane domains are underlined in blue. [The hydrophobic domain is conserved but the transmembrane domain is not].


10 20 30 40 50 60 70 80 90 100 110 120
hPCSK4 MRPAPIALWLRLVLALALVRPRAVGWAPVRAPIYVSSWAVQVSQGNREVERLARKFGFVNLGPIFPDGQYFHLRHRGVVQQSLTPHWGHRLHLKKNPKVQWFQQQTLQRRVKRSV-VVPT
rPCSK4 MRPSQTALWLGLVLSLALL---AVGWASARPPIYVSSWAVRVTKGYQEAERLARKFGFVNLGQIFPDDQYFHLRHRGVAQQSLTPHWGHRLRLKKEPKVRWFEQQTLRRRVKRSL-VVPT
mPCSK4 MRPSQTELWLGLTLTLALL---AVRWASAQAPIYVSSWAVRVTKGYQEAERLARKFGFVNLGQIFPDDQYFHLRHRGVAQQSLTPHWGHRLRLKKDPKVRWFEQQTLRRRVKRSL-VVPT
xPCSK4 -MPVWVVPRKSMITGPMVFVIFGLHWASSIK-IYTNSWAVHVPAGPDEVERITKKLGFINLGQVLHGSDLYHLQHRSVQKRSSSLHRSNGIQLKKEPMVHWFQQQTIKQRYKRQFKMVPT
130 140 150 160 170 180 190 200 210 220 230 240
hPCSK4 DPWFSKQWYMNSEAQPDLSILQAWSQGLSGQGIVVSVLDDGIEKDHPDLWANYDPLASYDFNDYDPDPQPRYTPSKENRHGTRCAGEVAAMANNGFCGVGVAFNARIGGVRMLDGTITDV
rPCSK4 DPWFSKQWYMNKEIEQDLNILKVWNQGLTGRGVVVSILDDGIEKDHPDLWANYDPLASYDFNDYDPDPQPRYTPNDENRHGTRCAGEVSATANNGFCGAGVAFNARIGGVRMLDGAITDI
mPCSK4 DPWFSKQWYMNKEIQQDLNILKAWNQGLTGRGVVISILDDGIEKDHPDLWANYDPLASYDFNDYDPDPQPRYTPNDENRHGTRCAGEVSATANNGFCGAGVAFNARIGGVRMLDGAITDI
xPCSK4 DPCFTKQWYLNNDVQPDLGVLTAWSQGYTGAGVVVTVLDDGIEKDHPDLSANYDPMASYDFNSNDPDPQPHYNPSDENRHGTRCAGEVAAAAFNNICGAGIAYNSRIGGIRMLDGIITDI
250 260 270 280 290 300 310 320 330 340 350 360
hPCSK4 IEAQSLSLQPQHIHIYSASWGPEDDGRTVDGPGILTREAFRRGVTKGRGGLGTLFIWASGNGGLHYDNCNCDGYTNSIHTLSVGSTTQQGRVPWYSEACASTLTTTYSSGVATDPQIVTT
rPCSK4 VEAQSLSLQPQHIHIYSASWGPEDDGRTVDGPGLLTQEAFRRGVTKGRQGLGTLFIWASGNGGLHYDNCNCDGYTNSIHTLSVGSTTRQGRVPWYSEACASTFTTTFSSGVVTDPQIVTT
mPCSK4 VEAQSLSLQPQHIHIYSASWGPEDDGRTVDGPGLLTQEAFRRGVTKGRQGLGTLFIWASGNGGLHYDNCNCDGYTNSIHTLSVGSTTRQGRVPWYSEACASTFTTTFSSGVVTDPQIVTT
xPCSK4 IEAQSLSLNPQHIHIYSASWGPEDDGKRVDGPGPLAQEAFFWGAVNGRGGLGSIFVWASGNGGMQYDNCNCDGYTNSIYTLSVGSTTEHGNVPWYSEACASTLTTTFSSGISTERKILTT
370 380 390 400 410 420 430 440 450 460 470 480
hPCSK4 DLHHGCTDQHTGTSASAPLAAGMIALALEANPFLTWRDMQHLVVRASKPAHLQAEDWRTNGVGRQVSHHYGYGLLDAGLLVDTA-RTWLPTQPQRKCAVRVQSRPTPILPLIYIRENVSA
rPCSK4 DLHHQCTDKHTGTSASAPLAAGMIALALEANPLLTWRDLQHLVVRASRPAQLQAEDWRINGVGRQVSHHYGYGLLDAGLLVDLA-RVWLPTKPQKKCTIRVVHTPTPILPRMLVPKNVTV
mPCSK4 DLHHQCTDKHTGTSASAPLAAGMIALALEANPLLTWRDLQHLVVRASRPAQLQAEDWRINGVGRQVSHHYGYGLLDAGLLVDLA-RVWLPTKPQKKCAIRVVHTPTPILPRMLVPKNVTA
xPCSK4 DIRMRCTDQHSGTSASAPLAAGIIALALEANPALTWRDLQHIVVRASNPSNLKAEDWSVNGVGRNVSHYYGYGLLDAGRNGGILXQKWQTAGVQRKCVVKFLNTPQVLSFNHVVRQTVDG
490 500 510 520 530 540 550 560 570 580 590 600
hPCSK4 CAG--LHNSIRSLEHVQAQLTLSYSRRGDLEISLTSPMGTRSTLVAIRPLDVSTEGYNNWVFMSTHFWDENPQGVWTLGLENKGYYFNTGTLYRYTLLLYGTAEDMTARPTGPQVTS--S
rPCSK4 CCDGSRRRLIRSLEHVQVQLSLSYSRRGDLEIFLTSPMGTRSTLVAIRPLDISGQGYNNWIFMSTHYWDEDPQGLWTLGLENKGYYYNTGTLYYCTLLLYGTAEDMTARPQTPQVTSCAH
mPCSK4 CSDGSRRRLIRSLEHVQVQLSLSYSRRGDLEIFLTSPMGTRSTLVAIRPLDISGQGYNNWIFMSTHYWDEDPQGLWTLGLENKGYYFNTGTLYYYTLLLYGTAEDMTARPQAPQVTSRAR
xPCSK4 CAG--SSNYIQSLEHVQAKISLSYSRRGDLEISLISPMGTRSVLVALRPYDTSTEGYKDWTFMSTHTWDEKPQGTWTLTLVNKGDFTNTGVLHDFTLVLYGTDEDMMSRRIEHSVLS---
610 620 630 640 650 660 670 680 690 700 710 720
hPCSK4 ACVQRDTEGLCQACDGPAYILGQLCLAYCPPRFFNHTRLVTAGPGHTAAPALRVCSS-CHASCYTCRGGSPRDCTSCPPSSTLDQQQGSCMGPTTPDSRPRLRAAACPHHRCPASAMVLS
rPCSK4 ACAEGHRGAVPGKSLSPL--HCGRTLPHL-----QQAVVVALQPHTAASD----------QGTGQLSPSYHT------CSAA----------
mPCSK4 ACVQRDTEGLCQESHSPLSILAGLCLISS-----QQWWWLYSHPQQPVT----------EGQASCHPPVTP------AAAA----------
xPCSK4 ECVTRDHSGKCHECKSSFYVFGNLCLSYCPPKFFKTLKKVPRTNGKPQSSHFALICASCHPSCYMCKGYSANDCISCPSFSTYDETTSSCSQPSFP----RTHHSIPNTSHLPQSAVATL
730 740 750 760 770
hPCSK4 LLAVTLGGPVLCGMSMDLPLYAWLSR-ARATPTKPQVWLPAGT---------
rPCSK4 ----------
mPCSK4 ----------
xPCSK4 IVGVTIPLLCLFTLIFSWVRFFQTNRPASQPPSTVEMNILSGMSEEGATIQE

I.5.3.2. Biological relevance of PCSK4

The restricted expression of PCSK4 to the gonads and the high conservation of its sequence among tetrapods suggested the important functions this enzyme play in reproduction. This was confirmed when the *Pcsk4*^{tm1Mbi} mouse lacking PCSK4 was produced (Mbikay *et al.*, 1997). Though they exhibited no physical or behavioural abnormality, their fertility in terms of fertile mating and of average litter size was very much impaired, especially in the males (Mbikay *et al.*, 1997). This reduction, however, was not associated with any apparent spermatogenic defect. PCSK4-null sperm are motile, but their hyperactivated motility following capacitation is reduced. *In vitro*, they are less competent than wildtype sperm at fertilising eggs, and eggs fertilised by PCSK4-null sperm fail to develop to the blastocyst stage (Mbikay *et al.*, 1997).

Pcsk4^{tm1Mbi} female mice are mildly subfertile: their rate of productive mating is normal, but the average size of their litters is reduced. They are less responsive to gonadotrophins treatment, and their ovaries exhibit signs of impaired folliculogenesis, ovulation, and luteinisation (Tadros *et al.*, 2001). These results indicate that PCSK4 plays an important role in fertilisation, as well as in early embryonic development in mice. Its production late in spermiogenesis and its localisation on the plasma membrane overlying the acrosome make it a potential target in non-hormonal contraception, including immunocontraception.

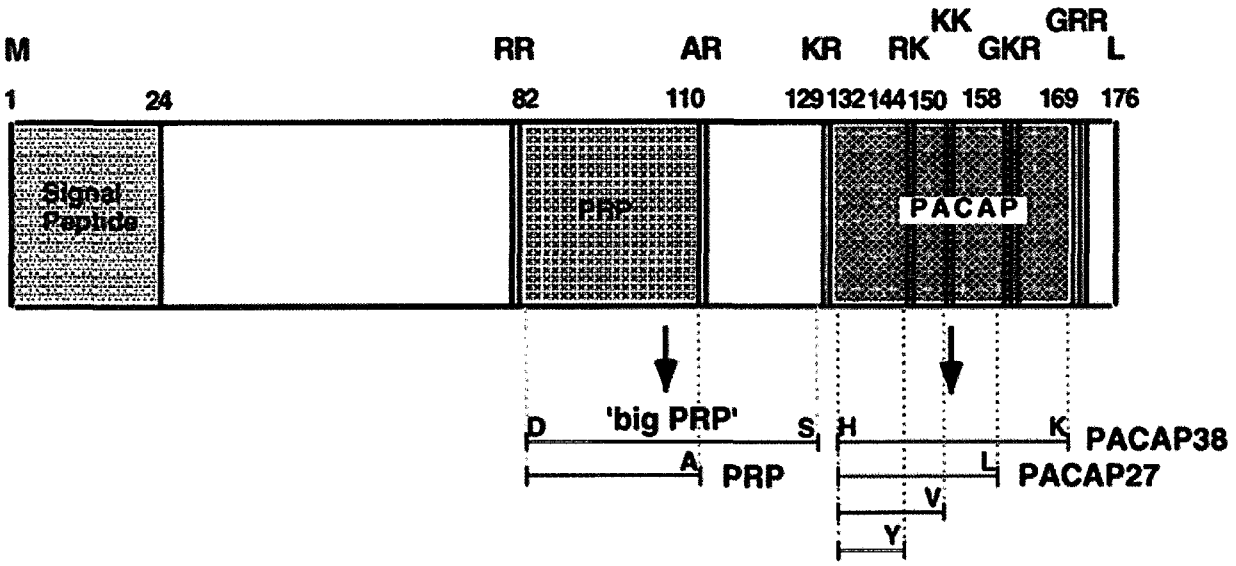
I.5.3.3. ProPACAP: A physiological substrate of PCSK4

Numerous precursor proteins produced in spermatogenic cells depend on PCSKs for processing. Among these are precursors to growth hormone-releasing hormone, secretin, gonadotropin-releasing hormone, pituitary adenylate cyclase-activating polypeptide (PACAP) (Li *et al.*, 1998), insulin-like growth factors (IGFs) I and II, (Gnessi *et al.*, 1997; Qiu *et al.*, 2005), IGF-I receptor (Naz and Padman, 1999), and hepatocyte growth factor receptor (c-Met) (Depuydt *et al.*, 1996).

PACAP is a member of the secretin/glucagon/vasoactive family of intestinal peptides. There are two amidated forms: PACAP38 (major form) and PACAP27, both originating from the same 126 amino acid precursor (Miyata *et al.*, 1989; Miyata *et al.*, 1990). It is very abundant in the brain and testis, but its levels in the testes exceed that in the brain (Miyata *et al.*, 1989; Miyata *et al.*, 1990; Arimura *et al.*, 1991). It shares about 68% sequence identity with Vasoactive Intestinal Polypeptide (VIP), but its ability to stimulate adenylyl cyclase is about 1000 times higher than VIP (Miyata *et al.*, 1989; Miyata *et al.*, 1990). Immunohistochemistry and *in situ* hybridisation histochemistry revealed PACAP-like immunoreactivity (PACAP-LI) and transcripts in round and elongated spermatids within the lumen of the seminiferous tubules; lower levels were present in spermatogonia and primary spermatocytes, but not in mature spermatids, testicular sperm, epididymal sperm, Sertoli cells, or Leydig cells (Shioda *et al.*, 1994; Yanaihara *et al.*, 1998). In spermatids, PACAP-LI was detected during the cap and acrosome phases of spermatids, but not in the maturation phase (Yanaihara *et al.*, 1998). *In situ* hybridisation also revealed that transcription of the *Pacap* gene occurs in developing germ cells, especially spermatogonia and primary spermatocytes (Kononen *et al.*, 1994; Shioda *et al.*, 1994; Hannibal and Fahrenkrug, 1995).

The sequence of proPACAP has a number of potential PCSK cleavage sites (Fig. 9), and the nearly simultaneous expression of *Pcsk4* mRNA and PACAP-LI in testicular germ cells strongly suggested PACAP as a potential physiological PCSK4 substrate. Li *et al.* confirmed this presumption when they reported that proPACAP is not processed into its active peptides, PACAP27 and PACAP38, in both the testes and ovaries of PCSK4-null mice (Li *et al.*, 1998; Li *et al.*, 2000b). They also reported that proPACAP is solely processed by PCSK4, and that, there were elevated levels of *Pacap* transcripts in the testes and ovaries of PCSK4-null mice as compared with those of the wildtype (Li *et al.*, 2000a). PACAP therefore becomes the first confirmed physiological PCSK4 substrate.

Figure 9. *Schematic model of processing pathway of PACAP precursor.* All possible cleavage sites for endoproteases are indicated by paired basic residues and multiple basic amino acids. M, R, D, A, S, K, H, Y, V, L, and G are the one-letter amino acid designations for amino acids Met, Arg, Asp, Ala, Ser, Lys, His, Tyr, Val, Leu, and Gly, respectively. Adapted from (Li *et al.*, 1998).


M
RR
AR
KR
RK
KK
GKR
GRR
L
1
24
82
110
129
132
144
150
158
169
176
Signal Peptide
PRP
PACAP
'big PRP'
D
S
H
K
PACAP38
A
PRP
L
PACAP27
V
Y

I.6. RATIONALE

The world population, which is growing at an alarming rate, has been projected to reach about 10 billion by 2050 (Kerr, 1995). The response from WHO, governments, and scientists to curb this growth was the introduction of hormonal contraception about five decades ago when women were called upon to bear the contraceptive responsibilities of their families (Schrager, 2002). Unfortunately, hormonal contraceptives pills, though effective, had disturbing and serious side effects (Schrager, 2002; Weber and Dohle, 2003). This gave impetus to the search for new and effective methods of contraception, preferably, for men (Waites, 2003). Vasectomy, condoms, and withdrawal (Martin *et al.*, 2000), though effective (Schwingl and Guess, 2000), are not acceptable by many men in different parts of the world. Moreover, vasectomy has disturbing side effects. ASA, also produced in vasectomised men, are a cause of infertility in humans (Diekman and Goldberg, 1994). This underscores the rationale for the development of immunocontraceptive vaccines. Owing to this, contraception strategies targeting sperm surface proteins which are involved in the functional maturation of sperm or in sperm-egg interaction are now strongly encouraged (Kerr, 1995; Herr, 1996; Naz, 1996; Frayne and Hall, 1999).

PCSK4 is a gonadal convertase. Its restricted expression to the gonads and the highly conserved nature of its sequence in mammals are indicative of the enzyme having an important biological role. Indeed, PCSK4 is involved in the cascade of events that lead to fertilisation, as male PCSK4-null mice are infertile. Its production late in spermiogenesis and its localisation on the sperm surface make it a potential target in the development of non-hormonal contraception, such as immunocontraception. Such non-hormonal contraceptives could be either inhibitors of PCSK4 enzymatic activity or its immunoblockers. A better understanding of the physiology and biochemistry of PCSK4 in fertilisation therefore will help determine, if it could be a good target for the rational design and application of non-hormonal contraception in mammals.

I.7. HYPOTHESIS

PCSK4 is a good target for immunocontraceptive.

I.8. OBJECTIVES

This thesis has been subdivided into three specific objectives in three manuscripts contained herein.

I.8.1. Investigating the contribution of PCSK4 to sperm fertilisation competence I

a. *To determine the subcellular localisation of PCSK4 in testis and sperm.*

b. *To investigate if PCSK4-null and wildtype sperm possess differential sperm capacitation rates.*

c. *To determine if PCSK4-null sperm possesses the same ability as wildtype sperm to disperse cumulus mass and bind to the egg's zona pellucida.*

Conventional immunohistochemistry, indirect immunofluorescence, and electron microscopy analyses were carried out on testicular sections or epididymal sperm from wildtype mice to study the subcellular location(s) of PCSK4. As a sequel to a previous report from our laboratory that PCSK4-null sperm are unable to fertilise eggs *in vitro*, and that eggs fertilised by them fail to develop to the blastocyst stage, sperm from PCSK4-null and wildtype mice were capacitated at 0, 30, 45, 60, 75, and 90 min and the extent of capacitation assessed by chlortetracycline assay. The ability of capacitated sperm from PCSK4-null and wildtype mice to acrosome react and to disperse cumulus masses were assessed; their ability to bind to the egg was also followed using egg-binding assay.

Charles Gyamera-Acheampong, Julierut Tantibhedhyangkul, Wattana Weerachatyanukul, Haidy Tadros, Hongbin Xu, Jan-W. van de Loo, R.-Marc Pelletier, Nongnuj Tanphaichitr,

and Majambu Mbikay (2006). **Sperm from mice genetically deficient for the PCSK4 proteinase exhibit accelerated capacitation, precocious acrosome reaction, reduced binding to egg zona pellucida, and impaired fertilising ability**. *Biol Reprod* 74, 666-673.

I.8.2. Investigating the contribution of PCSK4 to sperm fertilisation competence II

a. *To determine if PCSK4-null and wildtype sperm possess differential protein tyrosine phosphorylation rates.*
b. *To determine the component(s) of the capacitation medium responsible for observed hyper-tyrosine phosphorylation in PCSK4-null sperm and whether it is dependent on protein kinase A (PKA).*
c. *To determine the sperm proteins that are hyper-tyrosine phosphorylated in PCSK4-null sperm.*
d. *To study the proteolytic processing of ADAM2 and ADAM3*

The first manuscript reported that PCSK4-null sperm undergo maximal acrosome reaction at zona pellucida concentrations that are ineffective on the wildtype sperm, and that PCSK4-null sperm possess faster capacitation rate than wildtype sperm. Capacitation, a prerequisite for acrosome reaction, is associated with tyrosine-phosphorylation, thus tyrosine phosphorylation rates of the two sperm types were followed and compared. Proteolytic processing of ADAM2 and ADAM3, two critical sperm proteins involved in fertilisation, were also followed to verify if they are substrates for PCSKs, notably, PCSK4.

Charles Gyamera-Acheampong, Julian Vasilescu, Daniel Figeys, and Majambu Mbikay. **PCSK4-null sperm display enhanced protein tyrosine phosphorylation and ADAM2 proteolytic processing during *in vitro* capacitation.** *Fertility and Sterility: In press.*

I.8.3. Following the biosynthesis, maturation, and transport of PCSK4

a. *To investigate the mechanism of sperm surface localisation of mouse PCSK4.*

b. *To determine if HEK293 cells express PCSK4.*

c. *To study the biosynthesis and maturation of PCSK4 in HEK293 cells.*

d. *To investigate if HEK293 PCSK4 is enzymatically active.*

Mouse PCSK4 has no transmembrane domain yet it is located on the sperm surface. Through different extraction methods, it was established that the association is mediated through hydrophobic interactions. Conventional cloning techniques, transfections, pulse-chase analyses, and indirect immunofluoscence were used to follow the nature of the association. This led to the cloning of human PCSK4 (hPCSK4) from HEK293 cells. hPCSK4 biosynthesis, maturation, and enzymatic activity were then followed using conventional cloning and transfection techniques.

Charles GYAMERA-ACHEAMPONG, Francine SIROIS, Nicholas J. DENIS, Daniel FIGEYS, and Majambu MBIKAY. **ProPCSK4 is slightly matured to its active form in transfected somatic cells: evidence of interactions with BiP and of enzymatic activity.** *Manuscript revised and re-submitted to Biochimie on April 30, 2009.*

I.9. MANUSCRIPTS NOT INCLUDED IN THIS THESIS

I.9.1. Following other phenotype of the PCSK4-null mouse

To determine whether the body masses of PCSK4-null and wildtype mice are influenced by dietary fat

PERSONAL SERENDIPITOUS OBSERVATION: PCSK4-null mice become leaner than their wildtype litter mates as they age. Studies were conducted to follow the effect of dietary fat on body mass between the two congenics.

Charles Gyamera-Acheampong and Majambu Mbikay (2009). **Relevance of proprotein convertase subtilisin/kexin type 4 (PCSK4) in mammalian fertility (a review).** *Human Reproduction Update, 15(2):237-247.*

I.9.2. Circulating autoantibodies in normal sera of mammals

Charles Gyamera-Acheampong and Majambu Mbikay. **Circulating autoantibodies against PCSK4 and other convertases in normal sera of mammals.** *Manuscript in preparation.*

Manuscript is being prepared in close collaboration with Dr. Majambu Mbikay.

CONTRIBUTION: I designed the experiments and generated all the results in the manuscript.

I.9.3. Plasma PCSK9 levels

Janice Mayne, Angela Raymond, Anna Chaplin, Marion Cousins, Nadine Kaefer, **Charles Gyamera-Acheampong**, Nabil G. Seidah, Majambu Mbikay, Michel Chretien, Teik Chye Ooi. (2007). **Plasma PCSK9 levels correlate with cholesterol in men but not in women**. *Biochemical and Biophysical Research Communications* **361**: 451–456

CONTRIBUTION: I generated the antibody used in the study.

I.9.4. Pancreatic expression of 7B2

Gunther Schmidt, Francine Sirois, Younes Anini, Lisa M. Kauri, **Charles Gyamera-Acheampong**, Eckart Fleck, Fraser W. Scott, Michel Chrétien, and Majambu Mbikay. (2006). **Differences of Pancreatic Expression of 7B2 Between C57BL/6J and C3H/HeJ Mice and Genetic Polymorphisms at its locus (Sgne1)**. *Diabetes* **55**:452-459

CONTRIBUTION: I performed all experiments leading to Figs. 3A and 4B.

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II. MANUSCRIPT I: Sperm from mice genetically deficient for the PCSK4 proteinase exhibit accelerated capacitation, precocious acrosome reaction, reduced binding to egg zona pellucida, and impaired fertilising ability

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The manuscript was written in close collaboration with Dr. Majambu Mbikay.

CONTRIBUTIONS OF AUTHORS

Ms. Haidy Tadros contributed to Figures 1A and 2A; Dr. R.-Marc Pelletier contributed to Figure 1B; Ms Julierut Tantibhedhyangkul was involved in Figures 4 and 5; Dr. Wattana Weerachatyanukul performed the electron microscopy analysis; Dr. Jan-W. van de Loo provided the antibody; Mr. Hongbin Xu taught me sperm collection and analysis. I contributed to sperm collection for the electron microscopy analysis, all experimental work and statistical analysis leading to Figure 3, and all supporting experiments requested by reviewers that were not published as part of the manuscript. Dr. Nongnuj Tanphaichitr provided an intellectual contribution to the research presented herein and also critically reviewed the manuscript during its preparation.

We used conventional immunohistochemistry, indirect immunofluorescence, and electron microscopy to study the subcellular localisation of PCSK4 in testis and in sperm; Chlortetracycline assay was then used to investigate sperm capacitation rates between PCSK4-null and wildtype sperm that had been capacitated for 0, 30, 45, 60, 75, and 90 mins; the ability of capacitated sperm from PCSK4-null and wildtype mice to acrosome react and to disperse cumulus masses were then assessed, and their ability to bind to the egg was also followed using egg-binding assay.

The manuscript was submitted on August 24, 2005; revised and re-submitted on November 19, 2005; accepted for publication on December 12, 2005.

Sperm from Mice Genetically Deficient for the PCSK4 Proteinase Exhibit Accelerated Capacitation, Precocious Acrosome Reaction, Reduced Binding to Egg Zona Pellucida, and Impaired Fertilising Ability

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Short Title
PCSK4-null sperm capacitation and acrosome reaction

Keywords: Proprotein convertase, PCSK4, capacitation, acrosome reaction, egg binding fertilisation

Biology of Reproduction
December 21, 2005

ABSTRACT

The gene for proprotein convertase subtilisin/kexin type 4 (PCSK4, previously known as PC4) is primarily transcribed in testicular spermatogenic cells. Its inactivation in mouse causes severe male subfertility. To better understand the reproductive function of PCSK4, we examined its subcellular localisation in the testicular epithelium by immunohistochemistry, and on intact sperm by indirect immunofluorescence and immuno-electron microscopy. PCSK4 was detected in the acrosomal granules of round spermatids, in the acrosomal ridges of elongated spermatids and on the sperm plasma membrane overlying the acrosome. We also investigated PCSK4 relevance for sperm acquisition of fertilising ability by comparing wildtype and PCSK4-null sperm for their abilities in capacitation, acrosome reaction, and egg binding *in vitro*. PCSK4-null sperm underwent capacitation at a faster rate; they were induced to acrosome react by lower concentrations of zona pellucida; and their egg-binding ability was only half that of wildtype sperm. These sperm physiological anomalies likely contribute to the severe subfertility of PCSK4-deficient male mice.

INTRODUCTION

Proprotein convertase subtilisin/kexin type 4 (PCSK4, previously known as proprotein convertase 4 or PC4) belongs to a family of calcium-dependent serine proteinases that cleave secretory precursor proteins after selected pairs of basic residues. This family also includes PCSK1, PCSK2, PCSK3, PCSK5, PCSK6 and PCSK7 (previously known as PC1/3, PC2, furin, PC5/6, PACE4 and PC7/8, respectively). These enzymes are present in varying combinations in all cells. PCSK3, PCSK5, PCSK6 and PCSK7 are widely expressed. PCSK1 and PCSK2 are mostly found in endocrine and neuroendocrine cells [1, 2]. *Pcsk4* mRNA is readily detectable only in testicular germ cells [3-5].

PCSK4 is specified by a 9.5-kb, 15-exon gene located on mouse chromosome 10 (locus symbol: *Pcsk4*) and human chromosome 19 (locus symbol, *PCSK4*) [6, 7]. This gene is transcribed into a major mRNA isoform and several minor mRNA isoforms generated by differential splicing [3, 6]. *Pcsk4* transcripts are detectable by *in situ* hybridisation near the lumen of rodent testicular tubules [3, 8], and by Northern blot analysis, in testicular cell fractions enriched in spermatocytes and round spermatids [3]. They are absent in spermatogonia and in elongated spermatids, as well as in Sertoli and Leydig cells. The transcripts are first observed in mouse testis on postnatal day 16 (p16), after pachytene spermatocytes have appeared [9]. In the ovary, they are found in very low amounts in macrophage-like cells [9]. The major *Pcsk4* mRNA encodes a secretory precursor glycoprotein of 654 and 655 amino acids in rat and mouse, respectively [3, 4]. The encoded polypeptide is made up of an amino-terminal hydrophobic signal peptide, a prodomain, a subtilisin-like catalytic domain, a P domain and a carboxyl-terminal domain. Like all PCSKs, PCSK4 cleaves its substrates at the carboxyl side of an Arg, when this P1 Arg is preceded by another basic amino acid at P2 (Lys-Arg↓ or Arg-Arg↓) and/or at P4 (Lys-X-X-Arg↓ or Arg-

X-X-Arg↓, where X stands for any amino acid). Among PCSKs, PCSK4 is uniquely efficient at cleaving at Lys-X-X-Arg↓ sites [10, 11].

The restricted expression of PCSK4 suggested that this enzyme might play a role in reproduction. We have confirmed this presumption by producing the *Pcsk4*^{tm1Mbi} mouse lacking PCSK4 (–/–) [12]. Male fertility is severely reduced in these mice, both in terms of fertile mating and of average litter size. This reduction, however, is not associated with any apparent spermatogenic defect. PCSK4-null sperm are motile, but their hyperactivated motility following capacitation is reduced. *In vitro*, they are less competent in fertilising eggs and fewer of the fertilised eggs develop into viable embryos [12]. *Pcsk4*^{tm1Mbi} female mice are mildly subfertile: their rate of productive mating is normal, but the average size of their litter is reduced and their ovarian response to gonadotropin stimulation is diminished [9, 12].

The contribution of PCSK4 to sperm fertilisation competence is currently unknown. Freshly ejaculated mammalian sperm are incapable at fertilising eggs [13, 14]. However, as they travel in the female genital tract towards the ovulated egg, they attain fertilisation competence by undergoing a series of physiological, biochemical, morphological, and behavioural changes collectively termed as capacitation [15]. *In vitro* capacitation studies have unravelled some molecular events that take place during capacitation: among them, increased sperm metabolism [16, 17], changes in plasma membrane fluidity [18], changes in lectin reactivity [19-21], hyperactivated motility of sperm [15, 22], elevated intracellular pH [23], membrane hyperpolarisation [24], and increased protein tyrosine phosphorylation [25]. Fertilisation is achieved as receptors on the sperm head surface bind to ligands on the egg's zona pellucida (ZP). This binding triggers sperm intracellular signalling cascades that stimulate acrosomal exocytosis [26, 27]. Hydrolysing enzymes within the acrosome perforate the ZP matrix layer, paving the way for the sperm to enter into the perivitelline space.

Acrosome reacted sperm bind to the egg plasma membrane and one is incorporated into the egg proper after fusion of its plasma membrane with the egg plasma membrane [28].

In this study, we have attempted to gain a better understanding of the physiological role of sperm PCSK4 in fertilisation. Specifically, we examined the cellular and subcellular localisation of the PCSK4 protein in mouse spermatogenic and sperm cells; and, comparing wildtype and *Pcsk4*^{tm1Mbi} mice, we also investigated how lack of this enzyme affects sperm acquisition of fertilising ability.

MATERIALS AND METHODS

Animals and Antibodies.

CF-1 female mice from Charles River (Montreal, Quebec) and C57BL/6J (B6) male mice from our colony were used in this study. B6 mice were either wildtype (+/+) or null congenics (–/–) for the *Pcsk4*^{tm1Mbi} allele. They were housed in temperature-controlled rooms with 12-h dark-light cycles and were provided with food and drink *ad libitum*. They were handled according to the guidelines of the Canadian Council on Animal Care.

Two rabbit polyclonal anti-PCSK4 antisera were generated in our laboratories. The first antibody (Belg-αPCSK4) was produced using as antigen a purified recombinant mouse PCSK4 fragment produced in *E. coli*; it is suitable for immunoblotting [12]. The second antibody (αPCSK4-606) was produced by immunising rabbits with a rat PCSK4-expression plasmid DNA; it is suitable for immunohistochemistry [29]. Horseradish-peroxidase (HRP)-conjugated goat antibody against rabbit IgG was purchased from Amhersham (Piscataway, MA); biotinylated antibody against rabbit IgG, fluorescein isothiocyanate (FITC)-conjugated goat antibody against rabbit IgG and HRP-conjugated streptavidin were purchased from Molecular Probes (Eugene, OR); 15-nm gold coupled goat antibody against rabbit IgG was purchased from EY Laboratory (San Monteo, CA).

Collection and Processing of Gonadal Tissues and Cells

For testis histology, male mice were anaesthetised and perfused with Bouin fixative; testes were collected and processed as described elsewhere [30, 31]. For other studies, mice were sacrificed under anaesthesia by cervical dislocation. Sperm were collected from cauda epididymis and vas deferens (heretofore referred to as sperm) into Krebs Ringer bicarbonate (KRB)-HEPES medium (112.4 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4,

1.2 mM Mg_2SO_4, 4 mM $NaHCO_3$, 21 mM HEPES, 25 mM sodium lactate, 1 mM sodium pyruvate, 5.6 mM glucose, and 28 μM phenol red) containing 0.3% bovine serum albumin (KRB-HEPES-0.3% BSA).

Superovulation was induced in CF-1 female mice by intraperitoneal injection of 5 IU pregnant mare's serum gonadotropin (eCG) in the middle of the light cycle, followed by 5 IU of human chorionic gonadotropin (hCG) 48-50 h later. Cumulus-free eggs were prepared as described by Hogan *et al.* [32]. Ovarian zonae pellucidae (ZP) were isolated and solubilised as described by Tanphaichitr *et al.* [33].

Western Blot Analysis

Testis extracts were prepared by homogenisation and sonication in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA). Protein concentrations were determined by the Bradford dye-binding method [34] using reagents and a protocol from Bio-Rad Laboratories (Mississauga, ON). The extracts were analysed by Western blotting using the Belg-αPCSK4 antibody (1:1000 dilution) and HRP-conjugated goat anti-rabbit IgG antibody (1:2000 dilution) as primary and secondary antibodies, respectively. The antigen-antibody complex was detected using a Western Lightning Chemiluminescence Reagent Plus Kit (Perkin-Elmer, Boston, MA), following the manufacturer's instruction.

Immunohistochemistry

Endogenous peroxidase activity in testicular sections was inhibited with 0.6% H_2O_2 and immunolabelling conducted following a previously described protocol [35]. Briefly,

after blocking non-specific binding sites with 0.5% milk, the sections were successively incubated with the αPCSK4-606 antiserum (1:200 dilution), biotinylated anti-rabbit IgG (1:1000 dilution), HRP-conjugated streptavidin (1:200 dilution), and finally with 0.01% H_2O_2 and 0.05% diaminobenzidine tetrachloride (pH 7.7) for revelation. Control sections were incubated with the secondary antibody alone or with non-immune rabbit serum IgG. The stained sections were viewed under an Axiophot 2 Carl Zeiss microscope at 1,100x magnification. Photographs were captured on Technical Pan films from Eastman Kodak (Rochester, NY).

Indirect Immunofluorescence

Live sperm from wildtype and PCSK4-null mice were washed with phosphate buffered saline (PBS) by centrifugation at 350 *g*. They were then sequentially incubated for 30 min at room temperature, at ~10^7 cell/ml in PBS containing, first, 2% BSA to saturate non-specific antibody-binding sites; then αPCSK4-606 antibody (1:200 dilution); finally, with 25 μg/ml FITC-conjugated goat anti-rabbit IgG. They were washed with PBS following each incubation. Negative controls were sperm that were not treated with the primary antibody or were incubated with normal rabbit serum IgG instead of the primary antibody, but were processed otherwise like sperm treated with αPCSK4-606 antibody. An aliquot of the final sperm suspension was plated on a microscope slide and examined under a Zeiss Axioplan epifluorescence microscope (Carl Zeiss Canada, Mississauga, ON) at 500x magnification.

Immunogold Transmission Electron Microscopy (TEM)

Caudal epididymal sperm were collected into PBS. They were washed twice (350 *g*, 10 min) in the same medium. This medium was used throughout the whole procedure. Non-specific binding of antibody was blocked with 5% goat serum. Thereafter, the sperm were incubated with 4 μg/ml rabbit anti-PCSK4 IgG (αPCSK4-606) for 60 min at room temperature, washed, and then exposed to goat anti-rabbit IgG (1:100) coupled with 15-nm gold particles. After successive washes with PBS, sperm were fixed with 4% glutaraldehyde in PBS and routinely processed for embedding in LR-white resin (London Resin, Berkshire, UK). Negative control sperm were prepared in a similar manner to those described in the Indirect Immunofluorescence section. Ultra-thin sections mounted on nickel grids were counterstained with uranyl acetate and lead citrate prior to viewing under a Hitachi H-7100 transmission electron microscope at 75 kV.

Chlortetracycline (CTC) Assay for Sperm Capacitation

Sperm were collected from individual wildtype and PCSK4-null mice into KRB-HEPES medium containing no BSA. For standard capacitation, sperm pellets were carefully re-suspended in KRB containing 0.3% BSA to a density of 10^7 sperm/ml. Aliquots were immediately taken for zero time point chlortetracycline (CTC) staining. The rest were incubated for 90 min at 37 °C under 5% CO_2, and aliquots taken every 15 min for CTC staining assay. The assay was a slight modification [36] of the procedure described by Ward and Storey [37]. Briefly, 49.5 μl of sperm was added to 49.5 μl CTC working solution [500 - 750 μM CTC-HCl (Sigma), 130 mM NaCl, 5 mM cysteine, 20 mM Tris-Cl, pH 7.8] and incubated in the dark at room temperature for 1 min. The sperm were fixed by adding 1 μl of

25% glutaraldehyde in 2.5 M Tris-Base (1:1); 8 µl were spotted on a microscope slide, covered with coverslips, and placed in the dark at 4°C. A minimum of 200 sperm per slide were microscopically analysed within 24 h using the FITC filter on Zeiss Axioplan 2 Imaging (Carl Zeiss Canada, Mississauga, ON), and three slides were prepared for each sperm sample. Sperm were scored according to A, B, or AR patterns as described by Lee and Storey [38]. Pattern A (also referred to as pattern F) consisted of fluorescence over the entire head and midpiece region: it identified non-capacitated sperm; pattern B consisted of fluorescence-free band in the postacrosomal region: it identified capacitated acrosome intact sperm; pattern AR consisted of very low fluorescence over the entire head, except for bright fluorescence along the midpiece region: it identified capacitated acrosome reacted sperm. Percentages of sperm of the three CTC staining patterns were averaged from the three slides analysed on each experimental day. The experiment was conducted with 4 separate mice of each genotype on different days and the final percentages of sperm with various CTC staining patterns were calculated as means ± S.D.'s of the mean values from the four experiments.

ZP-Induced Acrosome Reaction

On each experimental day, sperm collected from a wildtype male and a PCSK4-null male were pre-capacitated in KRB-BSA as described above. They were then washed by centrifugation at 600 g and resuspended in KRB supplemented with 0.01% BSA at 10^6 sperm/ml. Solubilised mouse ZP were added to this sperm suspension to the final concentration of 2-8 ZP/µl. The sperm suspension was then incubated at 37°C under 5% CO_2 for 60 min to induce the acrosome reaction. They were washed, fixed in 4%

paraformaldehyde, treated with 0.04% Coomassie brilliant blue G-250 in 3.5% perchloric acid, applied to a slide and then viewed under a Zeiss Axioskop light microscope at 400x magnification. Acrosome intact and reacted sperm were differentiated by the presence and the absence of an intense blue stain of the acrosomal ridge of the acrosome, respectively. Two hundred sperm in various fields were assessed in each slide for their acrosomal status. Three replicate slides were made for each sperm sample. The average percentage of acrosome reacted sperm from the three slides in each experiment was recorded. The experiment was repeated four times and the final percentage of acrosome reacted sperm for each sample was expressed as a mean ± S.D. of the mean values from the four experiments.

In Vitro Sperm-ZP Binding

The assay for *in vitro* sperm-ZP binding was performed as described previously [33]. Approximately, 20-25 cumulus free eggs were incubated (37°C, 30 min, 5% CO_2) with 6 x 10^4 sperm of wildtype or PCSK4-null mice in a 60-μl droplet of KRB-BSA. One to two droplets of gamete co-incubates were set up for each sperm type. Subsequently, sperm-egg complexes were washed gently in 3 fresh KRB-BSA droplets through a drawn Pasteur pipette with a bore size of 200 μm in diameter to remove loosely attached sperm. They were then placed into a well of a sera culture slide, overlaid with mineral oil and examined under an inverted phase contrast microscope. Since numerous sperm bound to the egg ZP, only those observed in the same focal plane of the egg diameter were counted. To determine the percentage of sperm that bound non-specifically to ZP, two-cell embryos were co-incubated with sperm and washed under the same conditions. Based on the number of bound sperm per 2-cell embryo, only 5% of sperm bound to the egg ZP could be considered non-specific. The

sperm-ZP binding assay was performed 3 times on different days and the results were expressed as means ± S.D.'s of the average numbers of sperm bound per egg on the three different days.

In Vitro Fertilisation

Cumulus masses enclosing approximately 15-40 ZP-intact eggs, retrieved from oviducts of superovulated mice, were incubated (37°C, 5% CO_2) with 10^6 motile PGC sperm of wildtype or PCSK4-null mice in 1 ml of KSOM (95 mM NaCl, 2.5 mM KCl, 1.7 mM $CaCl_2$, 0.35 mM KH_2P0_4, 0.2 mM Mg_2SO_4, 25 mM $NaHCO_3$, 10 mM sodium lactate, 0.2 mM sodium pyruvate, 0.2 mM glucose, 1 mM glutamine and 0.01 mM EDTA) supplemented with 0.1% BSA. One to two dishes of sperm-cumulus mass co-incubates were set up for each sperm type. Dispersion of cumulus cells was monitored at 100x magnification under an inverted Nikon Diaphot microscope, as a function of time after insemination (i.e., 0, 1.5 and 9 h). At 9 h, the number of eggs containing two pronuclei (evidence of fertilisation) was determined, and the IVF rate was expressed as percent eggs fertilised with total number of eggs inseminated defined as 100%. Experiments were repeated on 3 three different days.

Statistical Analysis

Differences between wildtype and PCSK-null sperm in ZP-binding and in IVF rate were analyzed for significance by Student's *t*-test.; differences of ZP-induced acrosome reaction were analyzed by ANOVA.

RESULTS

Temporal Expression and Subcellular Localisation of PCSK4

Pcsk4 transcripts are first detected in the testis on postnatal day 16 [9]. To determine when translation of these transcripts begins, we conducted an immunoblotting analysis of testicular extracts from mice of different postnatal ages. An immunoreactive band of ~54 kDa, presumably corresponding to the mature enzyme, was first detected on day 16 and became more abundant with age (Fig. 1A). A smaller form of ~45-kDa was also consistently observed.

To determine which testicular cell types express PCSK4, immunohistochemistry was performed on testis sections of wildtype mice. Strong PCSK4-specific staining was detected in the acrosomal granules of round spermatids and the acrosomal ridges of elongated spermatids (Fig. 1B, panels a-d, thin arrows). Spermatocytes contained minute PCSK4-positive granules (Fig. 1B, panels a, b and d, wide arrows). Surprisingly, PCSK4-specific staining was also observed in residual bodies engulfed by Sertoli cells at spermatogenic stages VIII and IX (Fig. 1B, panels b and c, arrowheads).

Indirect immunofluorescence analysis of live sperm from wildtype and PCSK4-null mice showed an intense PCSK4-specific fluorescent signal over the sperm head convex ridge of wildtype sperm (Fig. 2A, panel a), but not on that of PCSK4-null sperm (Fig. 2A, panel b). Finally, when immunogold TEM was performed on intact wildtype mouse sperm, gold particles were observed on the sperm plasma membrane overlying the acrosome (Fig. 2B, panels a and b). Control sperm revealed minimal gold deposition (Fig. 2B, panel c).

Figure 1A. *PCSK4 expression in the testis - Immunoblot analysis.* Immunoblot analysis of PCSK4 expression during ontogeny. The analysis was conducted using 20 μg of protein extracts and the Belg-αPCSK4 at 1:1000 dilution.

1A

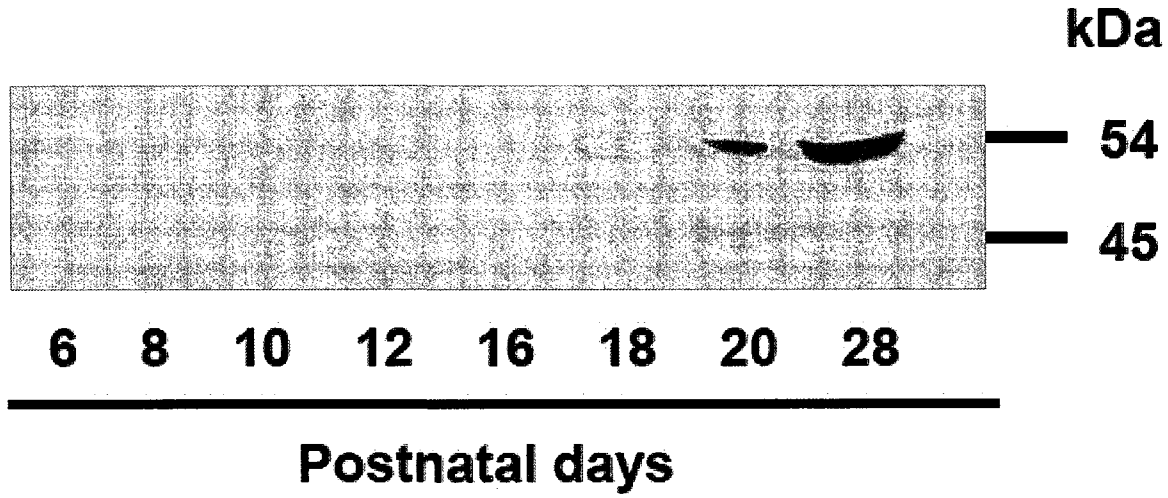


Figure 1B. *PCSK4 expression in the testis - Immunohistochemical analysis.* Immunohistochemical analysis of testicular sections of 3-months old mice for PCSK4 expression. Thin arrows, round and elongated spermatids; wide arrows, spermatocytes; arrowheads, residual bodies. Magnification, 1,100x. [Roman numerals represent different stages of mouse spermatogenesis].

1B

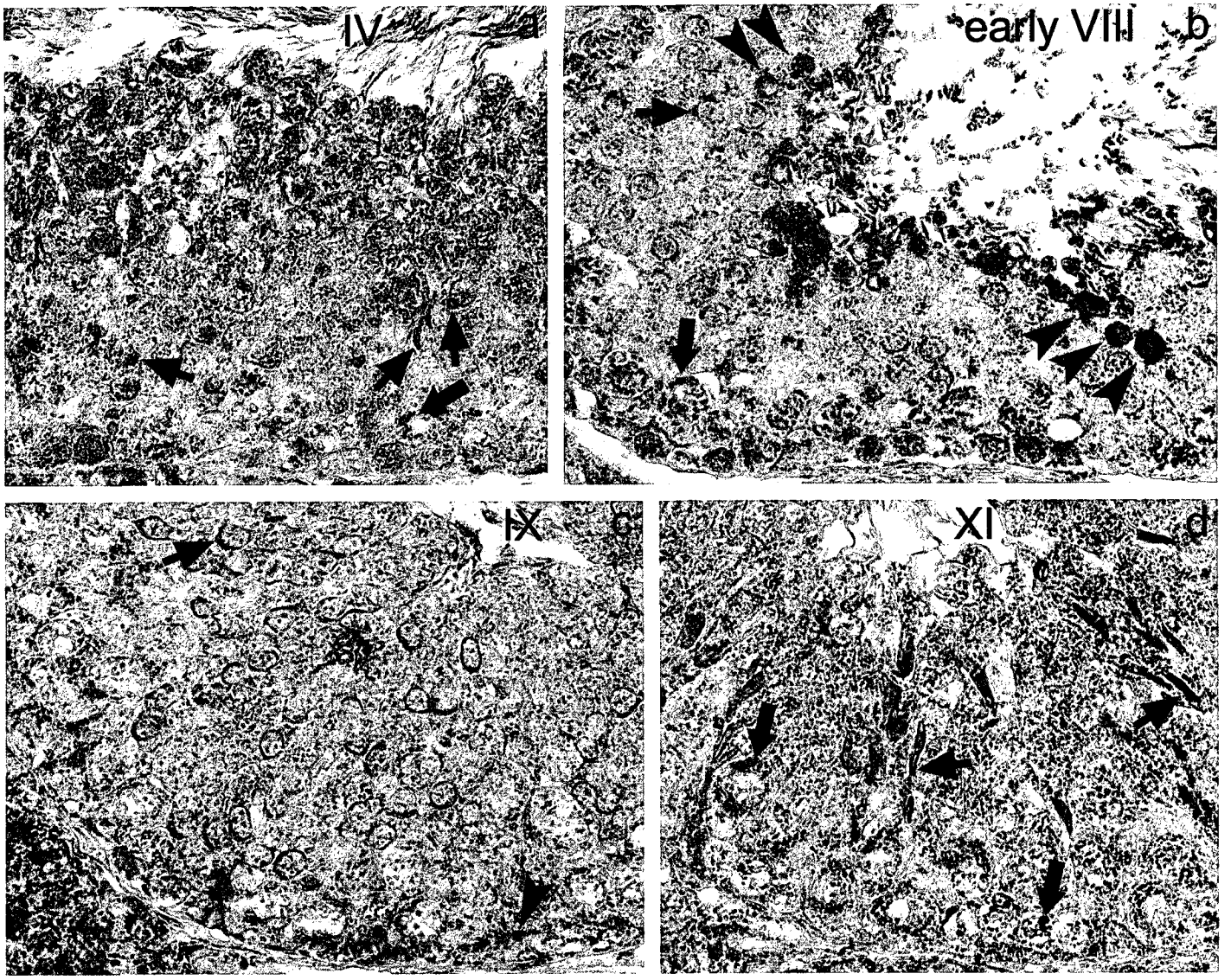


Figure 2A. *Localisation of PCSK4 on intact sperm using indirect immunofluorescence.* Indirect immunofluorescence analysis of intact mouse sperm: panels a and b sperm from +/+ and –/– mice, respectively.

2A

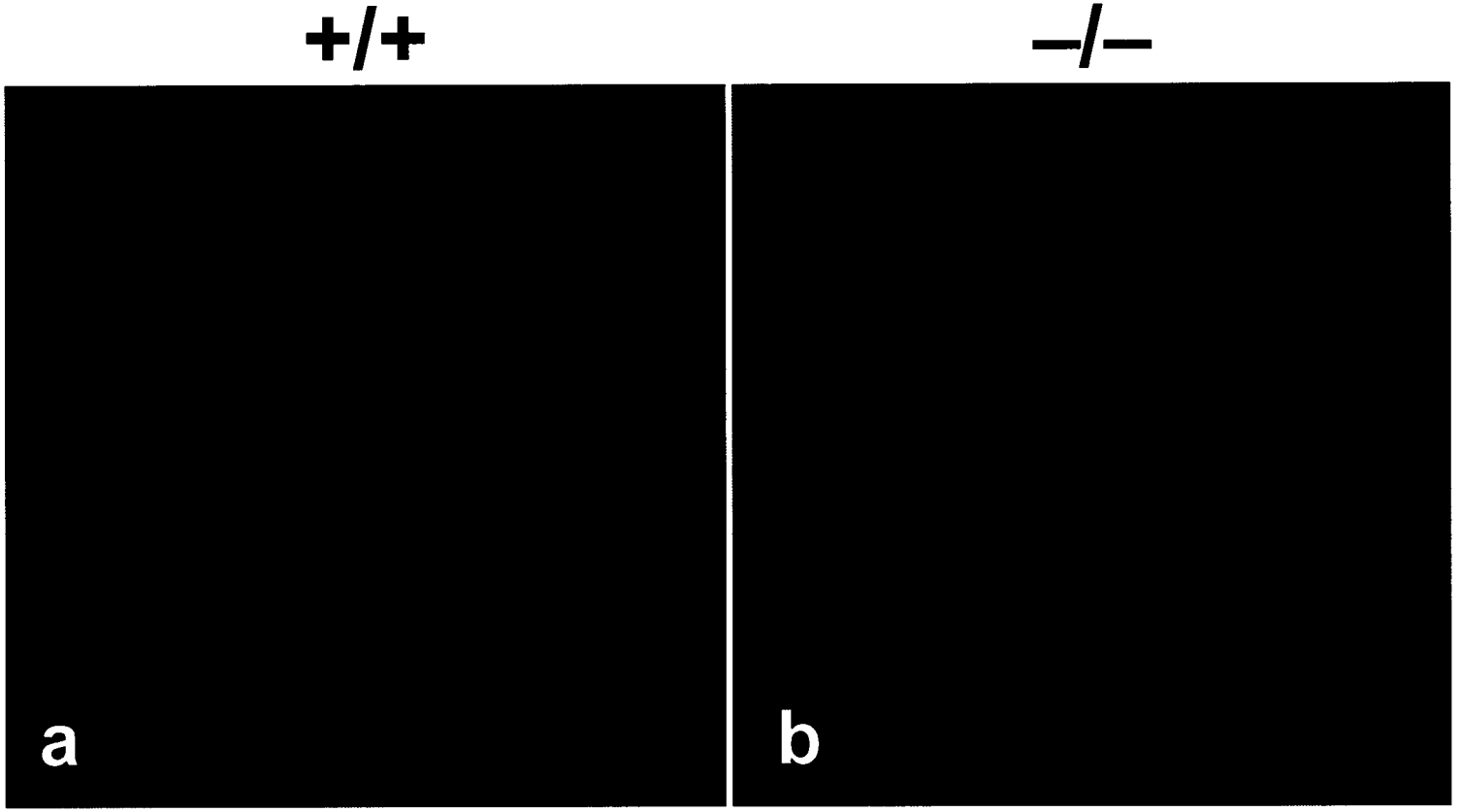
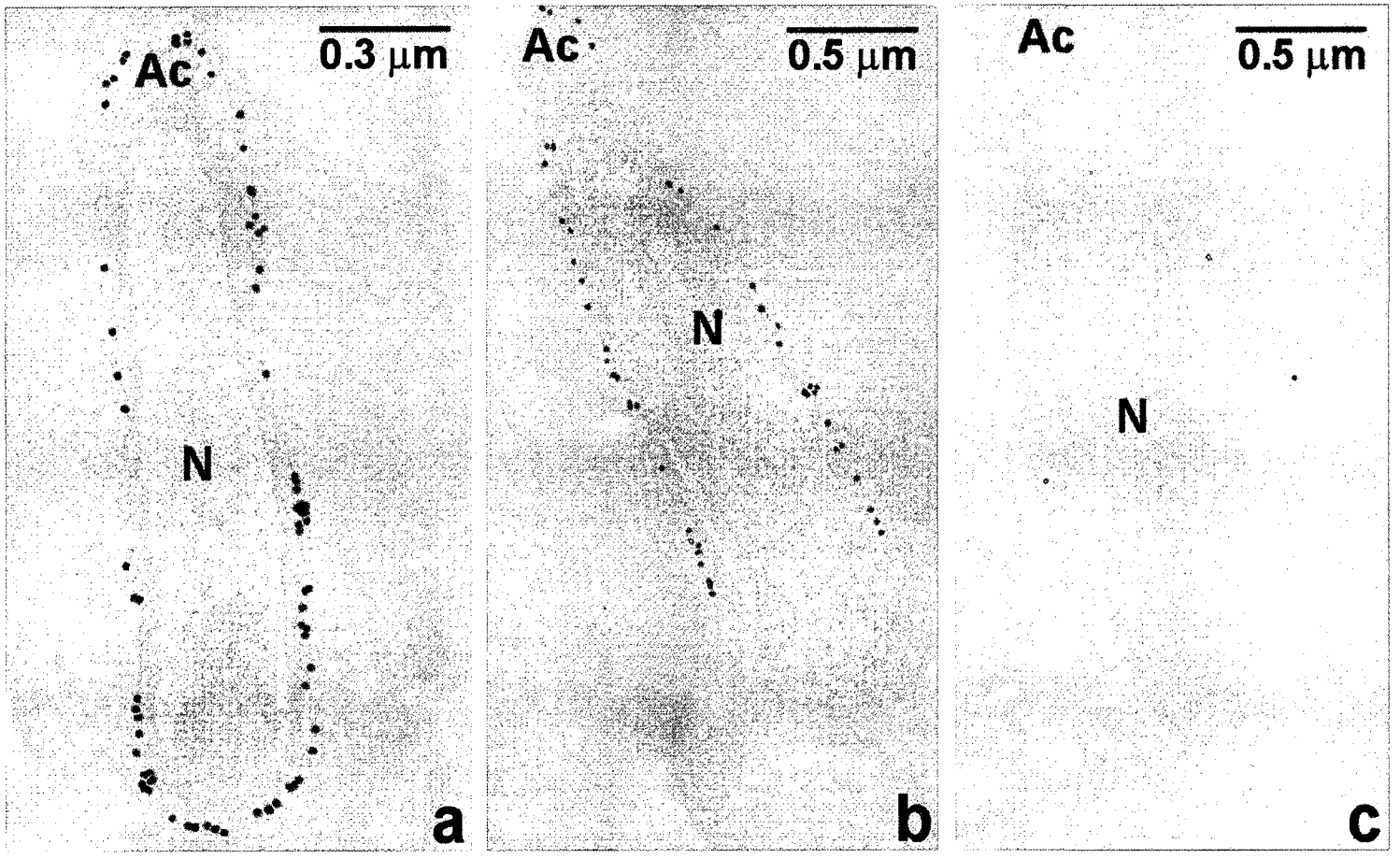


Figure 2B. *Localisation of PCSK4 on intact sperm using immunogold TEM.* Immunogold TEM localisation of live cauda epididymal sperm with anti-PCSK4 IgG (panels a and b). Panel a: horizontal section of the sperm head through the dorsal aspect of the acrosomal region, panel b: oblique section through the post-acrosomal region and the caudal end of the acrosome, panel c: for control, sperm was processed as in panels a and b, except that the primary antibody was omitted. N = nucleus, Ac = acrosome.

2B



Increased Rate of Capacitation of PCSK4-Null Sperm

In an effort to understand the reduced ability of PCSK4-null sperm to fertilise eggs *in vitro* [12], we examined the physiological steps that lead to sperm acquisition of fertilising ability. We first compared the rate of capacitation of wildtype and PCSK4-null sperm using the CTC staining technique. The results are shown in Fig. 3. During the initial 30 min, the rate of capacitation of PCSK4-null sperm was significantly faster than that of wildtype sperm. The time required for 50% of the sperm to be capacitated, but still remain acrosome intact, was 19 and 34 min for PCSK4-null and wildtype sperm, respectively. Furthermore, while about 80% of PCSK4-null sperm were of pattern B at 30 min, only about 28% of the wildtype sperm have gained that pattern. After 45 min, however, the level of capacitation reached a similar plateau of 85-90% for both sperm types. The rate of spontaneous acrosome reaction after 90 min was 10-20% for either sperm type.

Increased Sensitivity of PCSK4-Null Sperm to Zona-Induced Acrosomal Exocytosis

We also examined whether the ZP-induced acrosomal exocytosis differed between capacitated wildtype and PCSK4-null sperm. We first studied the time course of the acrosome reaction in the presence of 8 solubilised ZP/μl, a concentration commonly used to induce maximum acrosome reaction in mouse sperm, in 60 min at 37°C [39]. The percentage of reacted sperm (unstained by Coomassie brilliant blue at the head anterior ridge) was similar between wildtype and PCSK4-null sperm at all time points (Fig. 4A), indicating that the two types of sperm underwent the acrosome reaction with similar kinetics at this most effective ZP concentration. In a separate experiment, we studied the dependence of the acrosome reaction on ZP concentrations. Capacitated sperm from wildtype mice were incubated in medium containing ZP at 2, 4, 6 and 8 ZP/μl for 60 min.

Figure 3A. *Chlortetracycline (CTC) staining patterns.* Sperm were scored according to A, B, or AR patterns as described by [17]. *Pattern A* (also referred to as *pattern F*) consisted of fluorescence over the entire head: it identified non-capacitated sperm; *pattern B* consisted of fluorescence-free band in the postacrosomal region: it identified capacitated acrosome intact sperm; *pattern AR* consisted of very low fluorescence over the entire head, except for bright fluorescence along the midpiece region: it identified capacitated acrosome reacted sperm.

3A

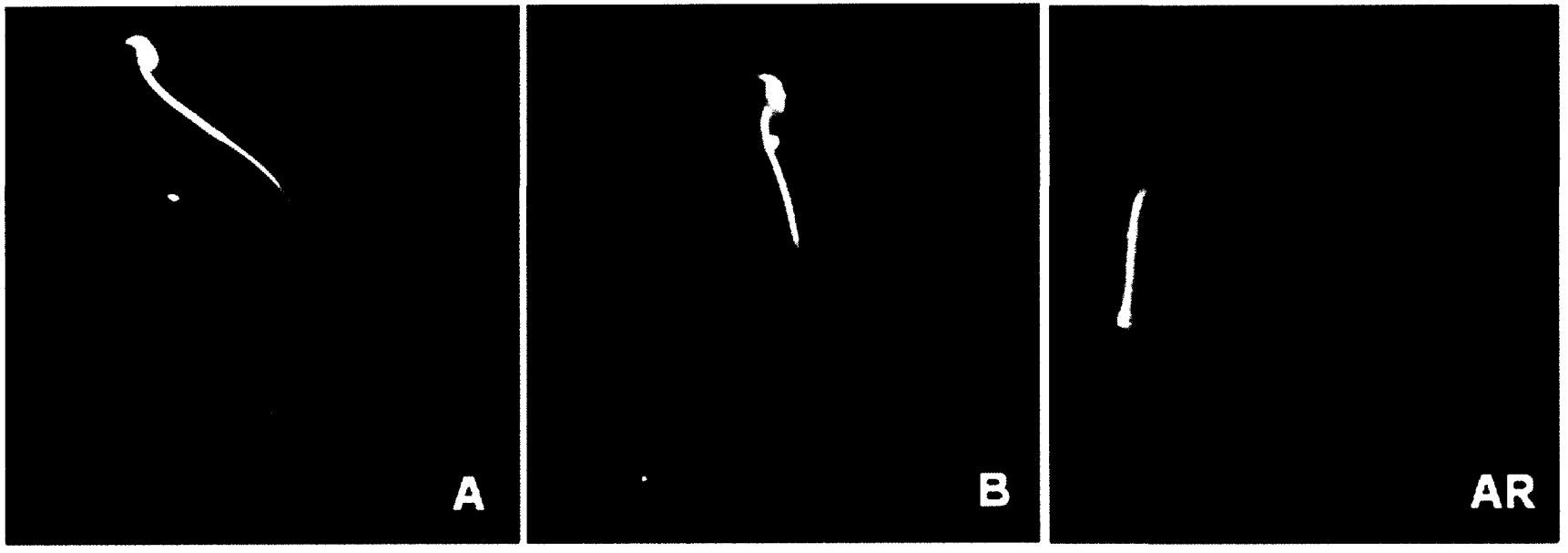


Figure 3B. *Kinetics of chlortetracycline assay.* CTC assay of the kinetics of in vitro capacitation of sperm from +/+ and -/- mice (n = 4). Rate of capacitation as line plot of the mean (±S.D.) percentage of sperm fluorescing in the A, B and AR CTC staining patterns with capacitation time. Symbols corresponding to each pattern for either genotype are identified in the inset.

3B

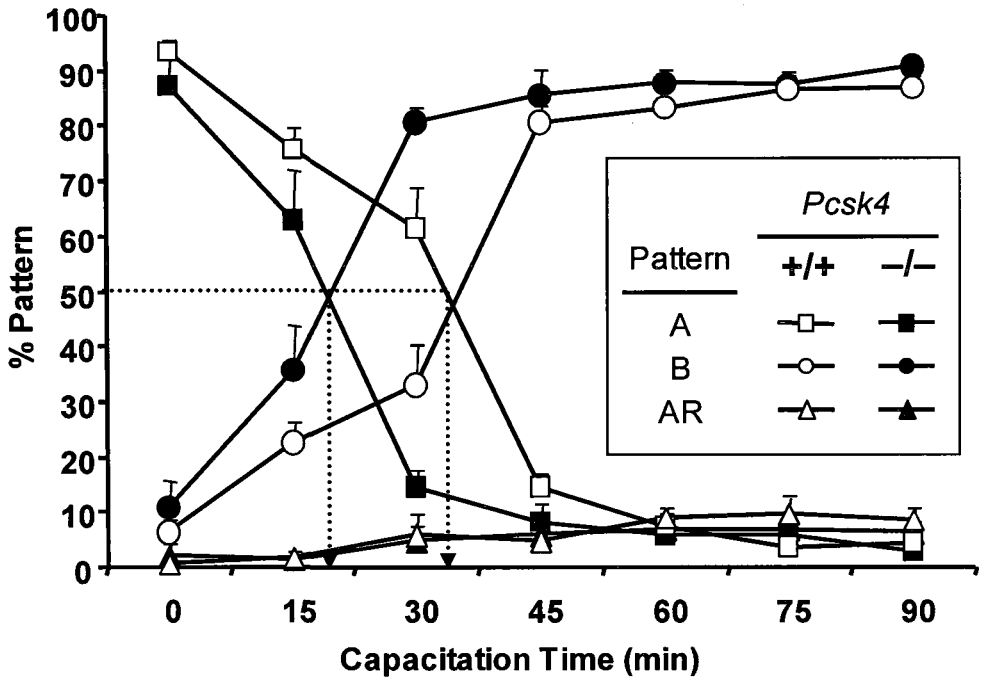
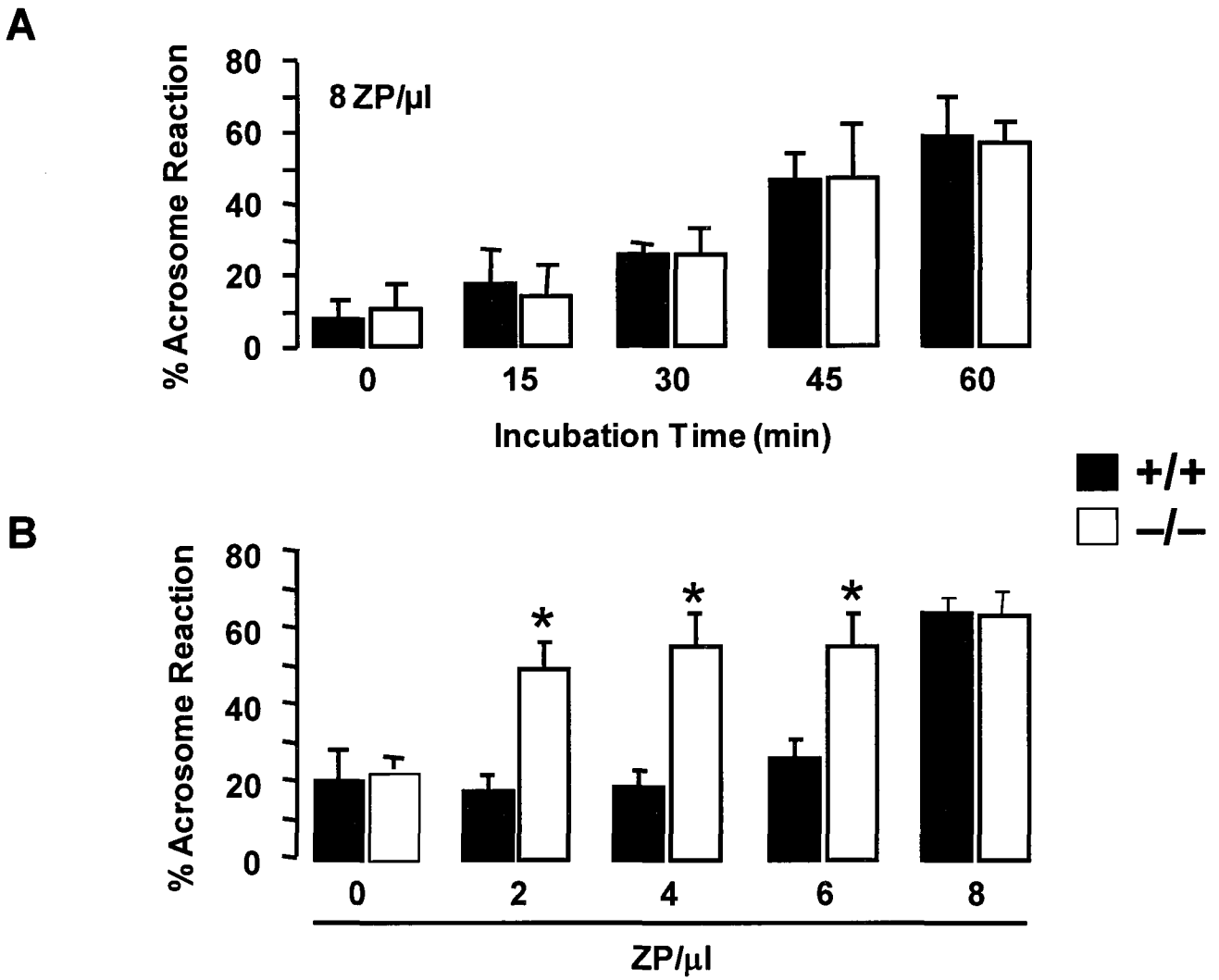

100
90
80
70
60
50
40
30
20
10
0
% Pattern
0
15
30
45
60
75
90
Capacitation Time (min)
Pcsk4
Pattern
+/+
–/–
A
B
AR

Figure 4. *Ability of wildtype and PCSK4-null sperm to undergo acrosome reaction.* These experiments were conducted on 4 separate occasions using 1 +/+ and 1 -/- mouse per experiment. In each sample, 200 sperm were assessed for acrosomal status and the percentage of acrosome-reacted cells was determined. The results are expressed as means ± SD of percent acrosome reaction for the 4 mice of each genotype. Black and white bars represent sperm from +/+ and –/– mice, respectively. **A)** Time-dependence of the acrosome reaction. **B)** Zona concentration-dependence of percent of acrosome-reacted sperm (AR) after a 60-min incubation. [Note: Differences at time "0" (spontaneous acrosome reaction) for **(A)** and **(B)** stem from variations between experiments.]


A
% Acrosome Reaction
80
60
40
20
0
8 ZP/µl
0
15
30
45
60
Incubation Time (min)
+/+
–/–
B
% Acrosome Reaction
80
60
40
20
0
*
*
*
0
2
4
6
8
ZP/µl

The rate of the acrosome reaction in the absence of ZP (spontaneous) was 10-20% for both sperm types. Capacitated wildtype sperm showed gradual increases in the percentages of acrosome reacted sperm following treatment with 2, 4, and 6 ZP/μl (18.0 ± 4.2, 18.8 ± 4.5 and 26.6 ± 5.1, respectively); this percentage then rose to 63.8 ± 6.5 in the presence of 8 ZP/μl. In contrast, when capacitated PCSK4-null sperm were treated with the lowest experimental concentration of 2 ZP/μl, nearly half of them (48.8 ± 6.8%) underwent the acrosome reaction. This percentage rose to 54 ± 9.0 when the ZP concentration was increased to 4 and 6 ZP/μl. Finally, it reached a level comparable to that of wildtype sperm (62.7 ± 10.0%) at 8 ZP U/μl (see Fig. 4B). These results suggested that PCSK4-null sperm were more sensitive to ZP-induced exocytosis.

Decreased Ability of PCSK4-Null Sperm to Bind to the ZP and to Fertilise Eggs in Vitro

The localisation of PCSK4 at the sperm surface overlying the acrosome, the site of ZP interaction, suggested that this proteinase may contribute to proteolytic events that facilitate fertilisation. To determine whether PCSK4 is necessary for penetration of sperm through the egg vestment, wildtype or PCSK4-null sperm were incubated with cumulus masses and the dispersion of these masses were monitored after 0, 1.5 and 9 h of incubation. No difference in cumulus cell dissociation was observed between the two sperm genotypes (Fig. 5A, panels a, c and e *vs* b, d, and f). However, after 9 h of incubation, no fertilised two-pronucleus egg was observed among eggs exposed to PCSK4-null sperm, whereas 40-51% of the eggs incubated with wildtype sperm were fertilised (Fig. 5A, panel g and Table 1).

The fertilisation incompetence of PCSK4-null sperm could be partly due to their inability to bind to ZP. Indeed, when egg-sperm-ZP binding assays were conducted using

capacitated wildtype or mutant sperm, the average number of bound sperm per egg was 13.4 ± 2.4 and 6.2 ± 4.0 for wildtype and PCSK4-null sperm, respectively ($n = 3$, $p < 0.001$) (Fig. 5B), an indication that lack of PCSK4 made sperm less efficient in egg binding.

Figure 5. *Ability of wildtype and PCSK4-null sperm to initiate and complete fertilisation.* **A)** Cumulus dispersion assay and fertilisation. Sperm from +/+ (panels a, c and e) and –/– (panels b, d and f) mice (n = 3) were incubated with cumulus intact cells; cumulus dispersion was microscopically assessed after 0 h (panels a and b), 1.5 h (panels c and d) and 9 h (panels e and f) of incubation. After 9 h, successful fertilisation was assessed by determining the percentage of eggs with 2 pronuclei (magnified in panels g *vs* h). Magnification: 100x. Bars: black, 500 μm; white, 100 μm; grey, 25 μm. **B)** Egg binding assay. Sperm were incubated with unfertilised, zona-intact eggs and the sperm-egg complexes microscopically examined. Black and white bars represent sperm from +/+ and -/- mice, respectively. Data were expressed as means ± S.D.'s of the average number of bound sperm/egg from three experiments. *, $P < 0.001$, n = 4.

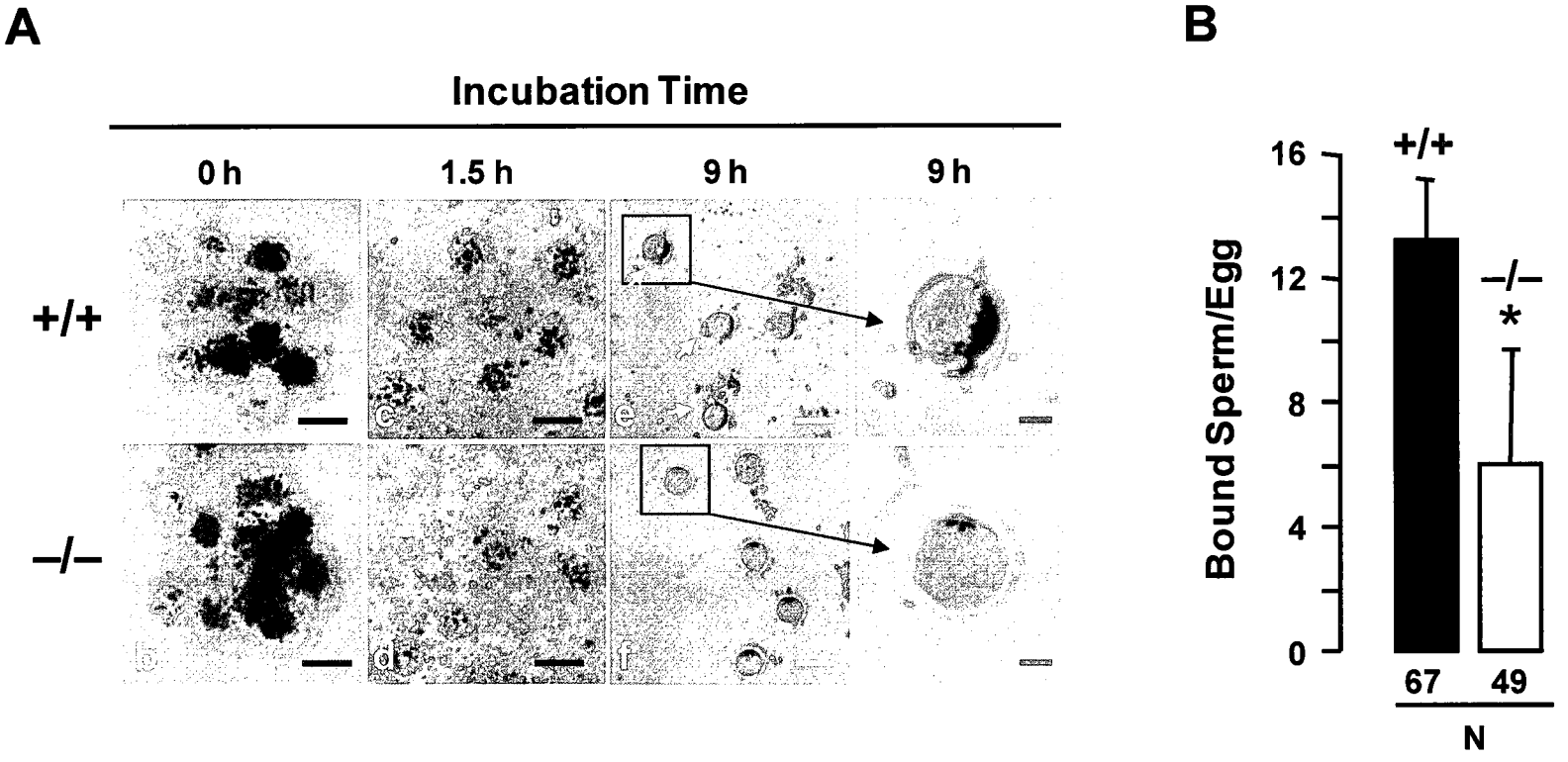

A
Incubation Time
0 h
1.5 h
9 h
9 h
+/+
–/–
B
Bound Sperm/Egg
16
12
8
4
0
+/+
–/–
*
67
49
N

Table 1. *Fertilisation Rate.* Number of two-pronucleus eggs per total number of eggs scored.

Pcsk4 Genotype	Experiment No		
	1	2	3
+/+	7/15 (47%)	19/37 (51%)	8/20 (40%)
-/-	0/21 (0%)	0/27 (0%)	0/21 (0%)

DISCUSSION

In a previous study of PCSK4 expression in neonatal mice, we reported that transcripts for this protein were first detected in the testis on postnatal day 16 [9]. Pachytene spermatocytes are known to appear in mouse testicular seminiferous tubules at this age [40]. In this study, we show by immunoblotting that PCSK4 immunoreactivity becomes detectable on the same day, indicating that transcription of the *Pcsk4* gene and translation of its mRNA are coordinated. This coordinated expression probably occurs in spermatocytes and in round spermatids only, as the *Pcsk4* mRNA virtually disappears in elongated spermatids [3, 5].

Based on their primary sequence and the presence of a single *N*-glycosylation site, proPCSK4 and PCSK4 are predicted to have molecular masses of ~70 and ~60 kDa, respectively. Two immunoreactive bands of apparent molecular masses of ~54 and ~45 kDa were observed in testicular extracts at the same postnatal ages. In a previous study using the same antibody, the 54-kDa form was shown to be absent in PCSK4-null sperm extracts [12], indicating that it is indeed PCSK4. The lower than expected molecular mass suggests that, like proPC1 [41, 42], proPCSK4 may be activated through multiple endoproteolytic events occurring at the amino (removal of the prodomain) and the carboxyl termini (truncation). Transduction in insect and mammalian cells of rat PCSK4 tagged with a V5 epitope at its C-terminus invariably leads to the loss of this epitope on the membrane-bound enzyme (unpublished data), supporting the likelihood of a C-terminal processing. The identity of the 45-kDa immunoreactive band is currently unknown. It may represent a PCSK4 degradation product or a variant protein encoded by one of the alternate *Pcsk4* mRNA isoforms found in the testis [3, 6].

Our immunohistochemical analysis has confirmed that PCSK4 expression in the

testis is restricted to the spermatogenic cells of the seminiferous tubules. PCSK4 immunoreactivity is most evident in the acrosomal granule of round spermatids and the acrosomal ridges of elongated spermatids. At some spermatogenic stages, it is also observed in residual bodies engulfed by Sertoli cells. Whether this immunoreactivity originates from active forms of PCSK4 or from their degradation products is still unclear.

Like all proprotein convertases [1, 2], PCSK4 is most likely produced in the endoplasmic reticulum as a secretory proprotein that is transported and modified through the Golgi cisternae before reaching its final destinations, including the acrosomal granule and the plasma membrane of the sperm head convex ridge. The association of mouse PCSK4 with the sperm plasma membrane was unexpected. Indeed, although the hydropathy plot of PCSK4 shows the presence of a conserved 20-amino acid hydrophobic domain ($GYYYNTGTLYYCTLLLYGTA^{559\text{-}578}$) in the carboxyl terminal region, this domain does not meet the standard criteria of membrane-spanning domains when tested using a variety of algorithms (http://us.expasy.org/). In contrast, a *bona fide* transmembrane domain has been identified in human and *Xenopus* PCSK4 [43]. We presume that rodent PCSK4 associates with the plasma membrane through its C-terminal 127-residue domain since a truncated form of rat PCSK4 lacking this domain is secreted into the culture medium when stably expressed in Drosophila Hi5 cells [11].

The localisation of PCSK4 at the sperm surface overlying the acrosome suggested that this protease may be implicated in proteolytic events associated with sperm maturation and capacitation, as well as fertilisation. The ability to undergo the acrosome reaction is one of the consequences of capacitation. Here, we show that, relative to wildtype sperm, PCSK4-null sperm are more reactive to capacitating conditions and zona-induced exocytosis. The molecular mechanism of this accentuated response is unclear. One can speculate that lack of

PCSK4 and impaired processing of its physiological substrates may cause qualitative, quantitative, and functional alterations in the signal transduction machinery of sperm. Spermatogenic cells produce a number of precursors to signalling molecules that depend on PCSKs for their processing. Among these proteins are precursors to growth hormone-releasing hormone, secretin, gonadotropin-releasing hormone, pituitary adenylate cyclase-activating peptides (PACAP) and insulin-like growth factors (IGFs) I and II [44], as well as IGF-I receptor [45] and hepatocyte growth factor receptor [46]. Of these precursors, only pro-PACAP has been shown to depend exclusively on PCSK4 for proteolytic activation, since its products, PACAP-38 and PACAP-27, are undetectable in the testis of PCSK4-null mice [47]. More recently, proIGF-II processing in placental cells was also found to be partly mediated by PCSK4 [29], suggesting that this enzyme plays the same role in the testis. PACAPs strongly activate mitogen-activated protein kinases (MAPK) of the extracellular signal-regulated kinase (ERK) types 1 and 2 in rat spermatids, through a mechanism involving cytosolic receptors [48]. PCSK4-null epididymal sperm contain reduced amounts of these kinases [48]. ERK-type MAPKs have been implicated in capacitation of human sperm [49-51]. How reduced expression of these kinases in PCSK4-null sperm correlates with the apparently accelerated capacitation displayed by these cells is unclear. Some insights may be gained by comparing the kinetics of activation of the MAPKs during in vitro capacitation of wildtype and mutant sperm. Pathways involving cAMP/protein kinase A pathway, membrane-bound phospholipase C and protein kinase C all contribute to sperm capacitation [52]. Their functionality in PCSK4-deficient sperm remains to be investigated.

It is highly probable that PCSK4 is present on the sperm surface as well as within the acrosome. Sperm surface PCSK4 is likely to be involved in a few steps of an early part of the fertilisation process. While PCSK4-null sperm could induce cumulus cell dispersion to the

same extent as wildtype sperm, they bound less efficiently to zona-intact eggs. The reduced egg-binding ability of PCSK4-null sperm may be caused by impaired proteolytic processing of sperm surface proteins that serve as ZP binding ligands. However, this reduced binding capacity may be offset by a higher ZP affinity of the receptors on the PCSK4 null sperm surface or by increased responsiveness of the downstream signalling pathway, resulting in precocious acrosome reaction as described in this report. On the other hand, PCSK4 in the acrosome, which is likely released during the acrosome reaction may be involved in ZP matrix digestion as all ZP glycoproteins contain several potential cleavage sites of PCSK4 in their amino acid sequences (NCBI Accession no. Q62005, NP_035905.1 and NP_035906.1, for mouse ZP-1, 2 and 3, respectively). This postulation is supported by our observation herein that PCSK4-null sperm had minimal ability to fertilise ZP intact eggs. In addition, it is also possible that PCSK4 deficiency leads to a relative decrease of several other sperm proteins involved in fertilisation-related steps. This phenomenon has been observed in calmegin, fertilin β, and cyritestin knockout mice [53-55]. Proteomics studies are under way to identify alterations of protein structure and protein-protein interactions in PCSK4-null sperm.

The total lack of fertilisation competence exhibited by the mutant sperm in this study is a more serious defect than the IVF inefficiency described in a previous report [12]. This discrepancy may be due to differences of genetic background: in the previous report, the mice were of a mixed B6-129Sv background; in this study, they were B6-congenics.

In conclusion, we have shown that PCSK4 is found on the acrosomal plasma membrane and that lack of this enzyme makes sperm more sensitive to capacitating conditions, more susceptible to the ZP-induced acrosome reaction, inefficient at binding to

zona pellucida and incompetent at fertilisation.

ACKNOWLEDGEMENTS

The authors thank Ms. Adrianna Gambarotta and Mr. Andrew Chen for their help in animal maintenance and surgery. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

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III. MANUSCRIPT II: PCSK4-null sperm display enhanced protein tyrosine phosphorylation and ADAM2 proteolytic processing during in vitro capacitation

Charles Gyamera-Acheampong, Julian Vasilescu, Daniel Figeys, and Majambu Mbikay. **PCSK4-null sperm display enhanced protein tyrosine phosphorylation and ADAM2 proteolytic processing during in vitro capacitation**. *Sterility and Fertility (In press).*

The manuscript was written in close collaboration with Dr. Majambu Mbikay.

Conventional approaches were used to verify if there are differences between PCSK4-null and wildtype sperm in terms of protein tyrosine phosphorylation; the role of PKA, Ca^{2+}, HCO_3^-, or albumin on tyrosine phosphorylation was also followed; the study also encompassed ascertaining if ADAM2 and ADAM3 are substrates for convertases, notably, PCSK4.

CONTRIBUTIONS OF AUTHORS

The LC-MS/MS analysis (Table 1) was contributed by Mr. Julian Vasilescu. I performed the sperm samples preparation, SDS-PAGE, and silver staining leading to the LC-MS/MS analysis. All other remaining aspects of this manuscript (tools generation, technical, experimental), were contributed by me. Dr. Daniel Figeys provided an intellectual contribution to the LC-MS/MS analysis presented herein. He also critically reviewed the manuscript during its preparation.

Fertility and Sterility (In press)

PCSK4-null sperm display enhanced protein tyrosine phosphorylation and ADAM2 proteolytic processing during in vitro capacitation

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Running title

Molecular alterations in PCSK4-null sperm

Capsule

PCSK4-null sperm proteins undergo enhanced tyrosine phosphorylation and more ADAM2 proteolytic processing during capacitation; thus alterations in signal transduction and proteolytic processing may underlie the fertilisation incompetence of PCSK4-null sperm.

Fertility and Sterility (In press)

ABSTRACT

Objective: To study the molecular basis for the accelerated capacitation rate in PCSK4-null sperm.

Design: Comparative and controlled experimental research study.

Setting: Academic medical institute

Animal(s): Male C57BL/6J wildtype or null congenics for the *Pcsk4* allele.

Intervention(s): Cauda and epididymal sperm were capacitated for varying times.

Main Outcome measure(s): Differences in sperm protein tyrosine phosphorylation and proteolytic processing of sperm egg ligands, ADAM2 and ADAM3.

Result(s): PCSK4-null sperm proteins are hyper-tyrosine phosphorylated during capacitation. This hyper-phosphorylation is dependent on PKA, albumin, and calcium. There is also more ADAM2 proteolytic processing from a 46-kDa form of ADAM2 to a 27-kDa form in PCSK4-null sperm than in WT sperm. This processing is dependent on cholesterol efflux.

Conclusion(s): Lack of PCSK4 is associated with quantitative changes in the phosphorylation and proteolysis of sperm proteins during capacitation; hence alterations in signal transduction and proteolytic processing during capacitation may underlie the fertilisation incompetence of PCSK4-null sperm. More investigation is needed to determine how and to what extent these changes might contribute to the loss of fertilising ability of PCSK4-null sperm.

Keywords: Proprotein convertase, PCSK4, capacitation, acrosome reaction, tyrosine phosphorylation, cholesterol efflux

INTRODUCTION

Proprotein Convertase Subtilisin/Kexin Type 4 (PCSK4), otherwise known as Proprotein Convertase 4 (PC4), belongs to a family of calcium-dependent serine endoproteases involved in post-translational modifications of precursor proteins (proproteins) in the secretory pathways (1-3). Other members of this family include PCSK1, PCSK2, PCSK3, PCSK5, PCSK6, PCSK7, PCSK8, and PCSK9 [also known as PC1/3, PC2, furin or PACE, PC5/6, PACE4, PC7/8, Subtilisin Kexin Isozyme-1/Site 1 Protease (SKI-1/SIP), and Neural Apoptosis Regulated Convertase-1 (NARC-1), respectively]. The family is subdivided into three subfamilies based on their structural similarity to bacterial and yeast proteases: PCSKs 1 to 7 belong to the kexin-like subfamily; PCSK8 to the pyrolysin subfamily, and PCSK9 to the proteinase K subfamily (2). PCSKs are biosynthesised as multidomain precursors made of a signal peptide, a prodomain, a catalytic domain, a P domain (except for PCSK9), and a variable carboxyl-terminal domain which sometimes contain a transmembrane (1, 2, 4). Kexin-like PCSKs cleave proprotein substrates after single or paired basic amino acids within the motif $(R/K)X_n(R/K)\downarrow$ where X stands for any amino acid except Cys or Pro, and n for number 0, 2, 4, or 6 of amino acids between the basic residues (1). PCSK8 and PCSK9 cleave after a hydrophobic and an acidic residue, respectively (4).

PCSK4 is encoded by a 9.5-kb, 15-exon gene (5) that maps to chromosome 10 in mouse (locus symbol, *Pcsk4*) and to chromosome 19 in human (locus symbol, *PCSK4*) (6). Its transcription in mouse and rat produces a 2.8-kb major mRNA isoform and 5 splice variants (5, 7, 8). Translation of the 2.8-kb mRNA in rats results in a 654 amino acid glycoprotein (7, 8). Excluding the carboxyl terminal domain, the PCSK4 sequence is highly

conserved between rodents and humans.

PCSK4 transcripts have been observed by hybridisation techniques in testicular spermatocytes and in round spermatids only (7). The protein is also detectable in these cells, but persists and accumulates in elongated spermatids and sperm; in sperm, it is found on the membrane overlying the acrosome (9). Low levels of *Pcsk4* transcripts are detectable by RT-PCR in mouse ovaries (10). In *Xenopus laevis*, the transcripts are predominantly found in the ovaries and testes (11). PCSK4 has also been reported to be present in human placenta (12).

PCSK4 was presumed to play a reproductive function because of its primary localisation in testicular germ cells and in sperm. This was confirmed in heritably PCSK4-null mice: the original mice of 129/Sv-C57BL6/J (B6) mixed genetic background exhibited no physical or behavioural abnormalities, but their fertility was impaired, more severely in the males, relative to heterozygotes (het) intercrosses: crosses of het females to null males resulted in only 25% successful mating and the average litter size was reduced by 88%; crosses of null females to het males resulted in 77% successful mating and the average litter size was reduced by 65%. In an *in vitro* fertilisation assay, the rate of egg fertilisation by PCSK4-null sperm was only 35% of that of wildtype (WT) sperm, and none of the eggs fertilised by the mutant sperm developed to the blastocyst stage (13). Our recent *in vitro* studies using B6 congenic mice have shown that PCSK4-null sperm undergo maximal acrosome reaction at concentrations of zona pellucida (ZP) that are ineffective on WT sperm. They are less efficient at binding to egg ZP, and are incapable of fertilising eggs (9). These studies also revealed that PCSK4-null sperm undergo capacitation at a much faster rate than WT sperm as demonstrated by the changing pattern of chlortetracycline stained acrosome (9).

In this study, we explored the molecular basis of the accelerated capacitation and the reduced egg-binding ability of sperm from PCSK4-null mice. We focused on sperm-protein tyrosine phosphorylation, a molecular signature of capacitation (14), as well as on the proteolytic processing of two "A Disintegrin And Metalloproteinase" (ADAM) proteins: ADAM2 and ADAM3. ADAM2 enables sperm to migrate from the uterus into the oviduct, bind to the egg's ZP, and attach to the egg's membrane (15). In contrast, ADAM3 is not required for migration into the oviduct but is required for the binding to the egg's ZP and plasma membrane (16, 17). Both proteins are known to result from limited proteolysis of larger precursors (16, 18, 19). Our results demonstrate that, during capacitation, PCSK4-null sperm undergo increased protein tyrosine phosphorylation and enhanced ADAM2 processing compared to WT sperm.

MATERIALS & METHODS

Sources of Chemicals and Bioreagents

Cholesterol sulphate (ChS) (Sigma, St. Louis, MO); decanoyl-Arg-Val-Arg-Lys-chloromethylketone (Dec-RVRK-CMK) and dec-Arg-Val-Lys-Arg-CMK (Dec-RVKR-CMK) (Bachem, Torrance, CA); dimethyl sulfoxide (DMSO) (Sigma); methyl-β-cyclodextrin (MBCD) (Sigma); *N*-[2-(*p*-Bromocinnamylamino)ethyl]-5-isoquinoline sulphonamide (H89) (Sigma); Elongase (Invitrogen, Burlington, ON); Lipofectamine Reagent (Invitrogen); QBI293A cells (a better adhering clone of the human embryonic kidney HEK293 cells; Qbiogene, Carlsbad, CA); Complete protease inhibitor cocktail (CPIC) (Roche Diagnostics, Mannheim, Germany); Protein A agarose (Sigma); anti-AKAP4 rabbit polyclonal antibody (pAb) (a gift from Dr. George L. Gerton, University of Pennsylvania, Philadelphia, PA); anti-V5 (the GKPIPNPLLGLDST epitope corresponding to residues 95-108 of simian virus 5 RNA polymerase alpha subunit) mouse monoclonal antibody (mAb) (Invitrogen); anti α-tubulin mAb (Sigma); anti-ADAM2 and anti-ADAM3 mAbs (Chemicon, Temecula, CA); horse radish peroxidase (HRP)-conjugated sheep anti-mouse IgG antibody (Amersham, Buckinghamshire, UK); anti-phosphotyrosine mAb (Clone 4G10, Upstate, Charlottesville, VA); RNeasy® Mini Kit (Qiagen, Mississauga, ON); Western Lightning Chemiluminescence Reagent Plus Kit (Perkin-Elmer, Boston, MA). The plasmid pIRES-hNARC1-V5-EGFP was a gift from Dr. N.G. Seidah of IRCM, Montreal, QC.

Animals

Mice used in this study were either male C57BL/6J (B6) WT or B6-*Pcsk4*^{tm1Mbi} congenics (PCSK4-null) (13). They were housed and fed as previously described (9), and were treated according to the Guidelines of the Canadian Council on Animal Care under a protocol approved by the institutional Animal Care Committee.

Sperm Collection and Capacitation

Mice were sacrificed under anaesthesia by cervical dislocation. Cauda epididymal and vas deferens sperm from both genotypes were collected into Krebs Ringer Bicarbonate (KRB)-HEPES medium (112.4 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM Mg_2SO_4, 4 mM $NaHCO_3$, 21 mM HEPES, 25 mM sodium lactate, 1 mM sodium pyruvate, 5.6 mM glucose, and 28 μM phenol red), containing 0.3% bovine serum albumin (KRB-HEPES-0.3% BSA), and subjected to a two-step Percoll gradient centrifugation (PGC) wash as previously described (20). The washed sperm were resuspended in KRB-0.3% BSA (KRB-BSA) to a concentration of 10^7 sperm/ml and incubated at 37°C with 5% CO_2 for up to 3 h to allow for capacitation. After capacitation, the percent motility, as assessed by duplicate visual count of the number of motile sperm over 100 sperm, ranged from 80% to 90% and statistically similar between the two genotypes and reproducible among experiments.

Immunoprecipitation and Immunoblotting

Unless stated otherwise, to extract proteins, tissues or cells were homogenised and sonicated in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 1%

NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing CPIC. Homogenates were centrifuged at 14 000 x g for 30 min at 4°C and supernatants collected. Protein concentrations in these extracts were determined by the Bradford dye-binding method (21) using reagents and protocol from Bio-Rad Laboratories (Mississauga, ON).

For immunoprecipitation (IP), human embryonic kidney QBI293A cell lysates were incubated on ice for 1 h with [1:250] dilution of mAb:V5. Protein A Agarose beads were then added to the antigen-antibody complexes and incubated overnight at 4°C on a rotary shaker. Beads were washed 5 times with RIPA buffer and after adding 2x Laemmli sample buffer freshly supplemented with 5% 2-Mercaptoethanol (βME), samples were heated at 99°C for 10 min and resolved by 8.5% Tricine SDS-PAGE.

For immunoblotting (IB), protein extracts of 3 x 10^5 sperm (per lane) were solubilised in 2x Laemmli sample buffer freshly supplemented with 5% βME. Mixtures were allowed to incubate for 15 min at room temperature (RT) and then heated at 95°C for 5 min. Solubilised proteins were separated by 8.5% Tricine SDS-PAGE and electrophoretically transferred onto a 0.45 μM nitrocellulose membranes (Bio-Rad). After two 5-min rinses with water, the membranes were incubated at RT for 1 h in PBS containing 5% skimmed milk (PBS/milk) to block non-specific binding sites. The membranes were then incubated overnight at 4°C in PBS/milk containing a diluted primary antibody. After five 5-min water rinses, the membranes were incubated for 60 min at RT with HRP-conjugated sheep anti-mouse IgG secondary antibody diluted to 1:5000 in PSB/milk. The membranes were washed twice for 5 min each in water, once in PBS containing 0.05% Tween 20 (PBST), and finally 5 times for 5 min each in water. They were then probed for HRP reaction using Western Lightning Chemiluminescence Reagent Plus Kit as specified by the manufacturer.

Chemiluminescence was captured on film for different exposure time periods. Densitometry was performed on Syngene's ChemiGenius^{2}XE Bio Imaging System (Cambridge, MA) using exposed films where all bands were of optical densities within the extended dynamic range of the instrument.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Analysis

SDS-PAGE silver stained gel bands were excised and subjected to in-gel tryptic digestion as previously described (22). Digestions were carried out for 16 h using sequencing grade trypsin from Promega (Madison, WI). Peptides from gel bands were extracted and reconstituted in 30 µl of 5% formic acid (FA). Peptides were then analysed by LC-MS/MS by loading them at 5 µl/min onto a 200 µm x 50 mm precolumn packed with 5 µm YMC ODS-A C18 beads (Waters, Milford, MA) using an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA). Following a desalting step, the flow was split and peptides were eluted through a second 75 µm x 50 mm column packed with the same beads, at approximately 0.2 µl/min using a 5 – 80% gradient of Acetonitrile with 0.1% FA (J. T. Baker, Northbrook, IL) for 1 h. The LC effluent was electrosprayed into an LTQ linear ion-trap mass spectrometer (Thermo-Electron, Gormley, ON), and MS/MS spectra were acquired in a data-dependent acquisition mode that automatically selected and fragmented the four most intense peaks from each MS spectrum generated. Peptide and MS/MS mass tolerances were set at 0.2 and 0.8 Da, respectively. MS/MS data were then analysed and matched to protein sequences in the NCBI database (nrdb) using the MASCOT database search engine (Matrix Science, London, UK) with carbamidomethyl as a fixed modification and oxidation as a variable modification.

Expression Vectors for PCSK4 and ADAM2

The cDNA for rat preproPCSK4 with a COOH-terminal V5 tag was cloned into *Nhe*I/*Xba*I-digested pCIneo (Promega) to generate pCIneo-rPCSK4-V5. To construct an expression vector for mouse preproADAM2, total RNA was extracted from mouse testis using the Qiagen RNeasy® Mini Kit; it was reverse-transcribed into cDNA using oligo(dT) primer; the cDNA fragment corresponding to the full-length mouse preproADAM2 was amplified by PCR using the Elongase polymerase and a sense (5'-gctagcgctcatgtggctcatcttgcttc-3') and an antisense (5'-agtaccggtgtctttggattcactttcacttt-3') oligonucleotide primers carrying a 5' *Eco*47III and *Age*I cleavage sites, respectively; after cleavage with these enzymes to generate cohesive ends, the amplicon was cloned into corresponding sites in the backbone of the pIRES-hNARC1-V5-EGFP bicistronic vector, after removal of the hNARC1 cDNA insert; thus generating the vector, pIRES-ADAM2-V5-EGFP, with an ADAM2 open reading frame (ORF) terminating after the V5-encoding sequence. The accuracy of the ORF was confirmed by sequencing.

Cell Culture and Transfection

For transfection, QBI293A cells were seeded at 8 x 10^5 cells/3.5-cm dish in 2.5 ml of Dulbecco's Modified Eagle's Medium (DMEM) with 10% foetal bovine serum (Invitrogen) at 37°C under 5% CO_2. At about 90% confluence, cells were transfected with a total of 2 μg of plasmid DNA consisting of 1 μg of the pIRES-ADAM2-V5-EGFP together with either pCIneo empty vector or pCIneo-rPCSK5-V5 using the Lipofectamine Reagent as instructed by the manufacturer. Two days later, cells extracts were prepared for immunoblotting or immunoprecipitation/immunoblotting.

Statistical Analysis

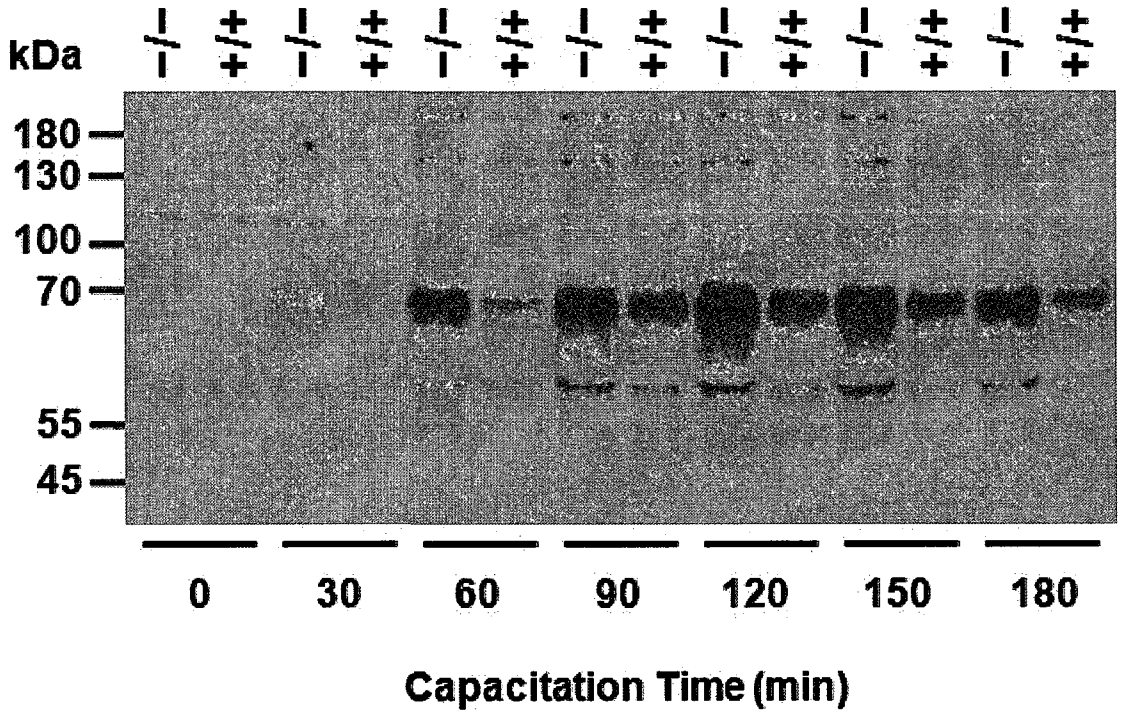
Differences between wildtype and PCSK4-null sperm in hyper-tyrosine phosphorylation during capacitation were analysed for significance by Student's *t*-test.

RESULTS

PCSK4-Null Sperm Proteins are Hyper-Tyrosine Phosphorylated During Capacitation.

We have previously reported that sperm from PCSK4-null mice undergo maximal acrosome reaction at concentrations of ZP that are ineffective on WT sperm and that they are incompetent at fertilising eggs *in vitro* (9). Since capacitation, a prerequisite for acrosome reaction, is generally associated with tyrosine-phosphorylation of sperm proteins (14), we decided to verify if PCSK4-null and WT sperm proteins undergo differential tyrosine phosphorylation during capacitation. PGC washed sperm from both genotypes were capacitated *in vitro* for 3 h. Aliquots were taken at different time points and analysed by immunoblotting with an anti-phosphotyrosine antibody. As shown in Fig. 1, tyrosine phosphorylated proteins ranging in size from ~45 to 250-kDa were observed in capacitated sperm of both types. However, the extent of phosphorylation, while being variable at 30 min, was consistently greater (4 to 5-fold, depending on experiments and mice) at and after 60 min in PCSK4-null than in WT sperm.

Figure 1. *A representative gel depicting how lack of PCSK4 leads to hyper-tyrosine phosphorylation in PCSK4-null sperm.* PGC washed sperm from both PCSK4-null and WT mice were capacitated for 0 – 3 h. Aliquots were taken every 30 minutes and immunoblotted for Yp-proteins using mAb:4G10 ("KO" or "-/-" represent PCSK4-null; "WT" or "+/+" represent wildtype).

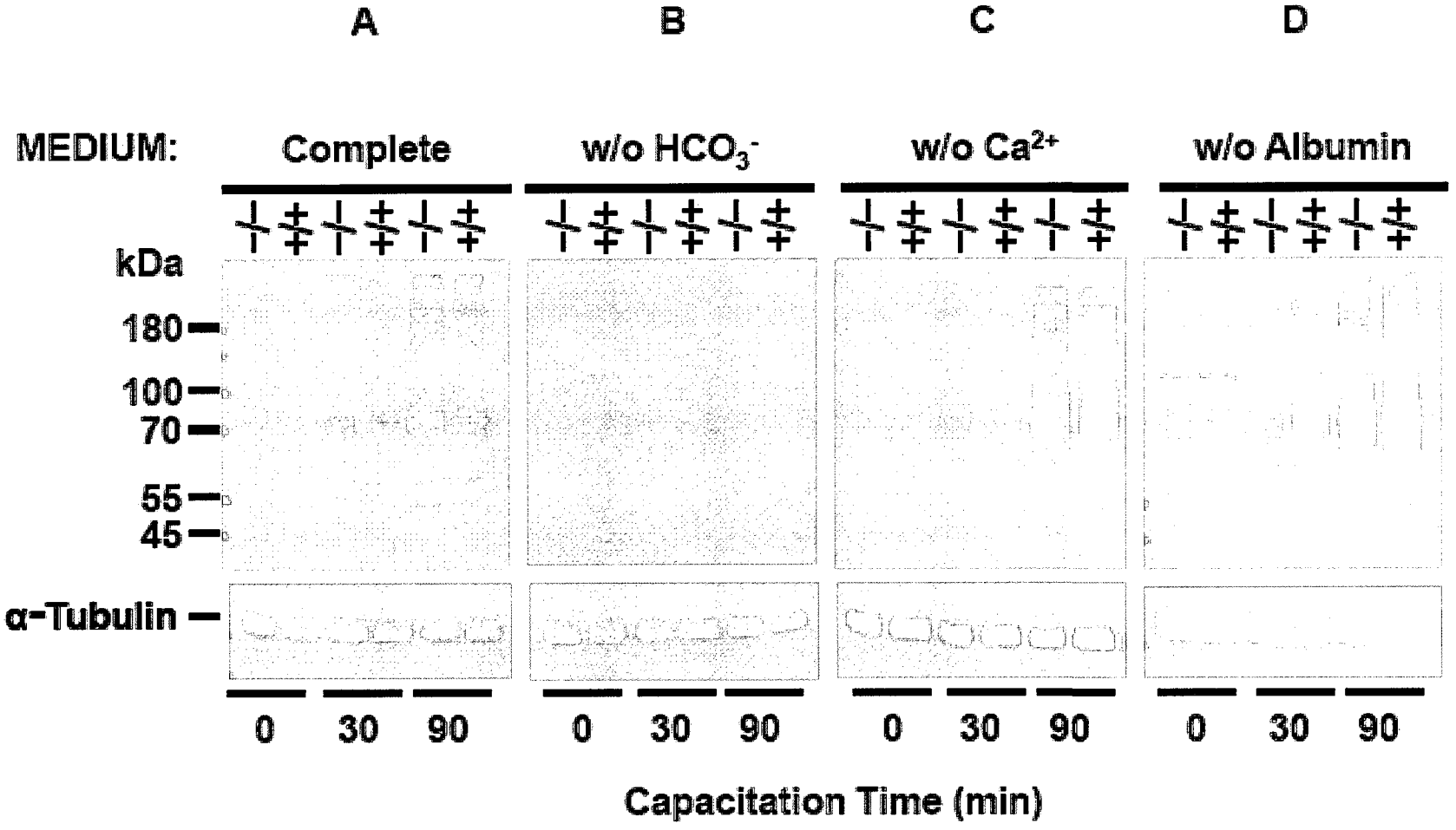

kDa
180
130
100
70
55
45
0
30
60
90
120
150
180
Capacitation Time (min)

PCSK4-Null Sperm Protein Hyper-Phosphorylation Is Albumin, Bicarbonate, and Calcium-Dependent

Media used for *in vitro* sperm capacitation generally contain albumin, Ca^{2+}, and HCO_3^- (14). Albumin is believed to remove cholesterol from the sperm plasma membrane probably accounting for observed sperm plasma membrane fluidity changes that occur during capacitation (14). It is unclear whether Ca^{2+} initiates and/or regulates capacitation, however a significant increase in intracellular Ca^{2+} from 70 to 250 μM during human sperm capacitation has been reported (14). Ca^{2+} is believed to act on sperm signal transduction effector enzymes such as adenylyl cyclase or cyclic nucleotide phosphodiesterase (14). The movement of HCO_3^- across the membrane could account for the reported increase in intracellular pH observed during capacitation (14). As mammalian sperm adenylyl cyclase is highly stimulated by HCO_3^-, this anion may serve to regulate sperm cAMP metabolism (14). The mechanism is however not fully understood.

In the next series of experiments, we sought to determine which of these three components affect tyrosine phosphorylation of PCSK4-null sperm proteins. The results in Fig. 2 demonstrate that phosphorylation in both PCSK4-null and WT sperm during capacitation requires HCO_3^- (Fig. 2B), but not Ca^{2+} (Fig. 2C) or albumin (Fig. 2D). However, in the absence of Ca^{2+} or albumin, the level of phosphorylation was comparable between PCSK4-null and WT sperm. These results suggest that complete capacitation medium is required to reveal differential tyrosine phosphorylation of sperm proteins from the two mouse genotypes.

Figure 2. *Effect of HCO_3^-, Ca^{2+}, and albumin on observed hyper-tyrosine phosphorylation in PCSK4-null sperm.* PGC washed sperm from both PCSK4-null and WT mice were capacitated for 0, 30, and 90 minutes in (A) complete capacitation medium; (B) capacitation medium devoid of HCO_3^-; (C) capacitation medium devoid of Ca^{2+}; (D) capacitation medium devoid of albumin. Sperm aliquots were immunoblotted for sperm proteins phosphorylated at tyrosine residues using mAb:4G10. In all cases, α-tubulin was used as house keeping protein.

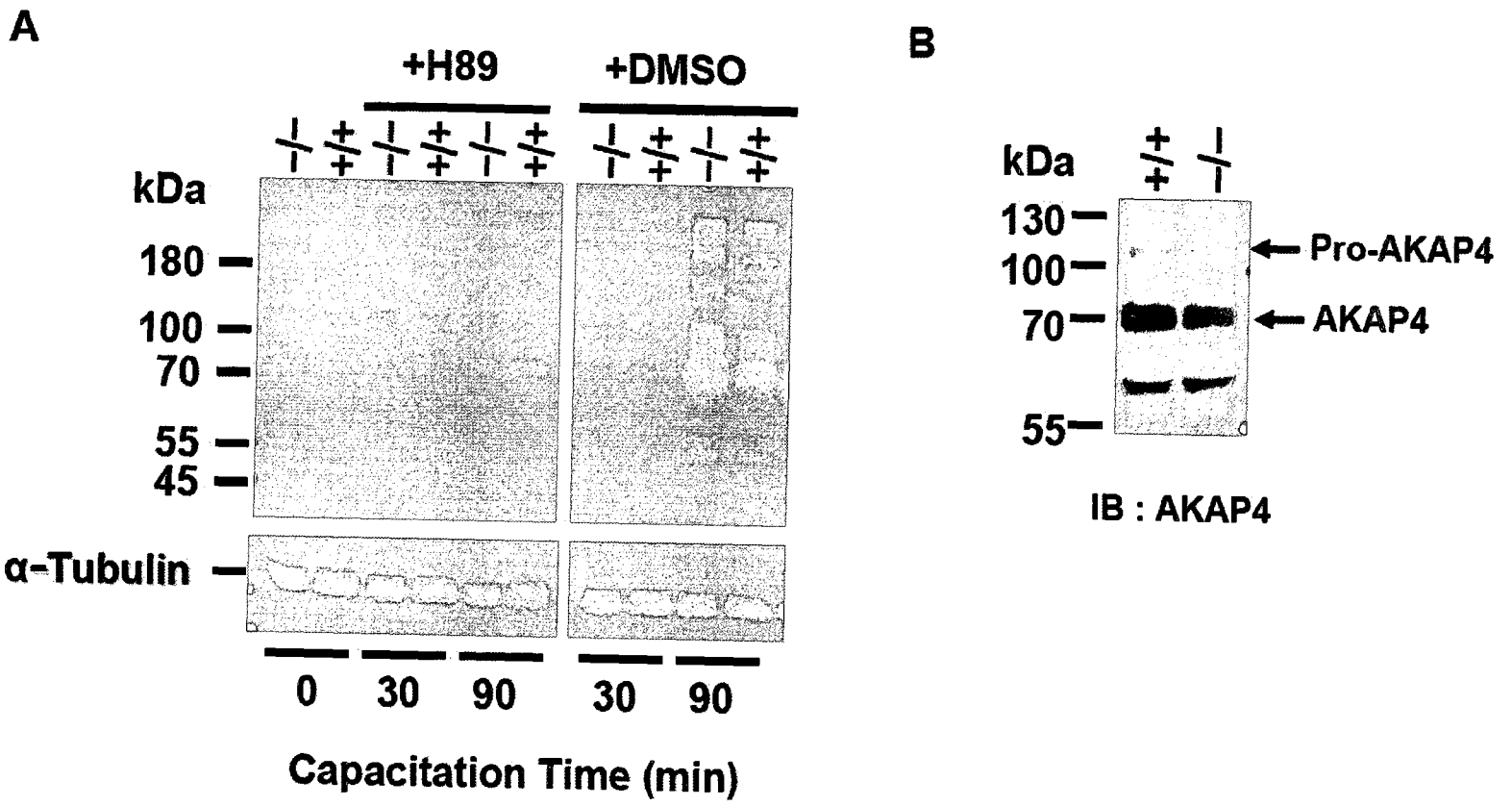

A
B
C
D
MEDIUM:
Complete
w/o HCO_3^-
w/o Ca^{2+}
w/o Albumin
kDa
180
100
70
55
45
α-Tubulin
0
30
90
Capacitation Time (min)

PCSK4-Null Sperm Protein Hyper-Phosphorylation Is PKA-Dependent

Protein Kinase A (PKA) regulates sperm motility through the cAMP-dependent phosphorylation of proteins in or associated with the fibrous sheath of sperm flagellum (23). To investigate if the differential increase in tyrosine phosphorylation occurs through the cAMP/PKA pathway, both sperm types were capacitated in the presence of H89, a potent and selective inhibitor of PKA (24). As shown in Fig. 3A, treatment with H89 reduced kinase activity such that there was less of it in phosphorylated proteins over time.

Mouse sperm flagellum has two major cytoskeletal components: the fibrous sheath (FS) and the outer dense fibres (ODF). A-Kinase Anchor Protein 4 (AKAP4), also known as AKAP82, is a FS protein and a key player in tyrosine phosphorylation of sperm proteins through its ability to bind to the regulatory (RII) subunit of PKA (25). Because lack of PCSK4 results in reduced hypermotility following capacitation (13), we examined by immunoblotting whether the accentuated tyrosine phosphorylation in PCSK4-null sperm was linked to altered AKAP4 expression in PCSK4-null sperm compared WT sperm. As previously reported (26), phosphorylated forms of pro-AKAP4 (~109-kDa) and AKAP4 (~75-kDa) were observed; their levels were comparable between the two types of sperm (Fig. 3B).

Figure 3. *Effect of H89 on observed hyper-tyrosine phosphorylation in PCSK4-null sperm.* **A)** PGC washed sperm from both PCSK4-null and WT mice were capacitated for 0, 30, and 90 minutes in complete capacitation medium supplemented with H89 or DMSO (control). Sperm aliquots were immunoblotted for sperm proteins phosphorylated at tyrosine residues using mAb:4G10. In all cases, α-tubulin was used as internal control. **B)** Sperm from PCSK4-null and WT mice were immunoblotted for AKAP4.


A
+H89
+DMSO
kDa
180
100
70
55
45
α-Tubulin
0
30
90
30
90
Capacitation Time (min)
B
kDa
130
100
70
55
Pro-AKAP4
AKAP4
IB : AKAP4

Mass Spectrometry of Proteins Co-Migrating in the 70-kDa Region

A significant proportion of tyrosine phosphoproteins (Yp-proteins) of capacitated sperm migrate on SDS-polyacrylamide gel with Mr of ~70-kDa (see Fig. 1-3). To identify by mass spectrometry the proteins that are differentially phosphorylated between PCSK4-null and WT capacitated sperm, we subjected extracts from both types of sperm to immunoprecipitation using the 4G10 monoclonal antibody. In spite of repeated efforts, sufficient material for mass spectrometry could not be obtained using this approach. To overcome this limitation, we fractionated capacitated sperm proteins from both types of sperm by SDS-PAGE, silver-stained the gel (27), excised the proteins that migrated at ~70-kDa region, digested the proteins with trypsin, and subjected them to LC-MS/MS analysis. The same proteins were identified in the two types of sperm. Table 1 lists proteins found in the gel fragments known to be tyrosine kinase substrates; it also summarises their presumed or established contributions to sperm fertilisation competence. Yp-proteins involved in post-capacitation sperm motility include AKAP3 (28), AKAP4 (29), and dihydrolipoamide S-acetyl transferase (30).

Proteolytic Processing of Testicular and Sperm ADAM2 and ADAM3

Since PCSK4-null sperm bind to egg ZP less than WT sperm (9), we presumed that the reduced binding could be due to lower levels of egg ligands at the surface of the mutant sperm. ADAM2 and ADAM3 are two such ligands (31). They are of particular interest in the context of this study because they are known to be biosynthesised as precursor proteins that must undergo a series of endoproteolytic maturation events to become active (18, 32). As these precursors contain potential cleavage sites for kexin-like convertases, we sought to determine whether lack of PCSK4 impairs their proteolytic maturation. We first analysed

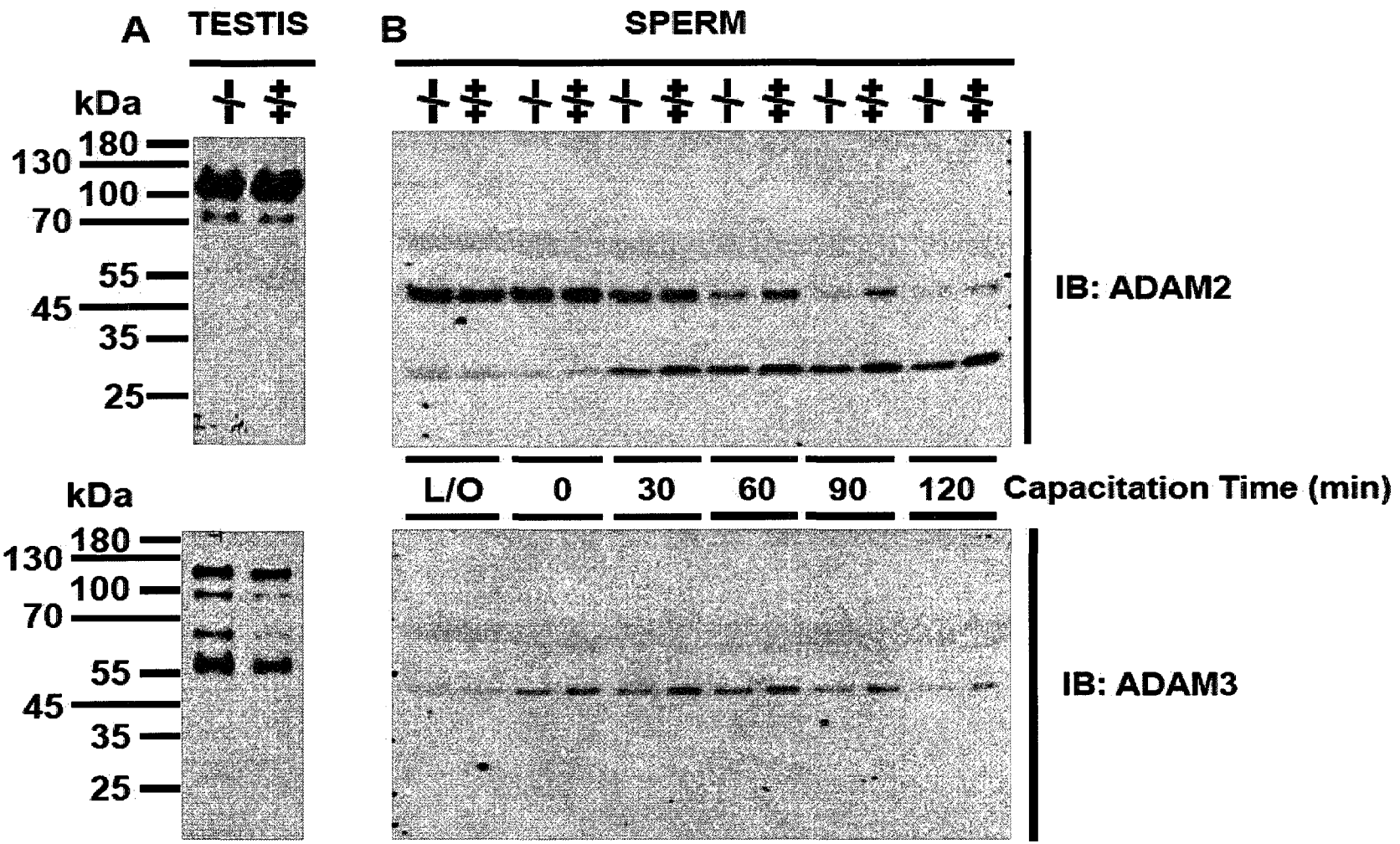
Table 1. *Proteins identified by LC/MS/MS previously known to be tyrosine-phosphorylated.* LC-MS/MS was used to identify 70-kDa proteins that are associated with sperm hyperactivated motility, tyrosine phosphorylation, capacitation, and acrosome reaction. gi#, GenBank accession number; PDE4A, phosphodiesterase 4A).

gi | #	Mass	Score	Peptides	Protein	Function
2290719	71404	761	KDKEVEELLQEIQCEKA RVTDLVNQQQSLEEKM RRQFQSQLADLQQLPDILKI KIDSLMNAVGCLKS	Outer Dense Fibre Protein	A major cytoskeletal structure of sperm tail involved in hyperactivated motility
47125065	68469	691	RVAPAPAGVFTDIPISNIRR KVPLPSLSPTMQAGTIARW KVPEANSSWMDTVIRQ KNFSAIINPPQACILAIGASEDKL	Dihyrolipoamide S-acetyltransferase Precursor	A component of pyruvate dehydrogenase S complex and is involved in sperm hyperactivation, tyrosine phosphorylation, capacitation, and acrosome reaction
20271173	95559	526	KYSNNGAALAELEEQAALVGSGSRC KEIVSDLIDSCMKN RLSSLVIQMARK KLLSESPFSCDELTESDNKR	AKAP4 (AKAP82)	Tethers PKA to fibrous sheath leading tyrosine phosphorylation of neighbouring proteins
6679939	48096	323	KNGQLVVDNLEINTYQCKD KLVAWYDNEYGYSNRV RVPTPNVSVVDLTCRL RDVVLTNVTVVQLRR	Glyceraldehyde-3-phosphate dehydrogenase S	Regulate flagellar movement
6753026	96780	175	RSVGEVLQSVLRY KFCEDEEATGGALSGLTKM KDTTIATILLKK KGTGTAEALLQNAYLTIHNELRG	AKAP3 (AKAP110)	Present in fibrous sheath and binds to PDE4A to negatively modulate c-AMP levels during sperm capacitation

testicular extracts from PCSK4-null and WT mice by immunoblotting for molecular forms of ADAM2 and ADAM3. We observed a major ADAM2 band of ~105-kDa corresponding to the precursor form, and two major ADAM3 bands of ~110-kDa and ~56-kDa, corresponding to the precursor and processed forms, respectively. These results are consistent with previous reports showing that proADAM3 processing begins in the testis (32), whereas that of proADAM2 occurs in the epididymis (18, 19). Band intensities did not significantly differ between PCSK4-null and WT mouse testicular samples. For both ADAMs, there were 2-3 minor immunoreactive bands which may represent intermediate processing products or proteins reacting non-specifically.

We also examined by immunoblotting the pattern of these ADAMs during epididymal sperm capacitation *in vitro*. The results are shown in Fig. 4B. Before capacitation, the sperm contained a single immunoreactive band of ADAM2 (~46-kDa) and ADAM3 (~50-kDa). Their levels were comparable in PCSK4-null and WT sperm. However, during capacitation, the ~46-kDa form of ADAM2 was gradually converted to a ~27-kDa form. Densitometry indicated that, before capacitation, the 27-kDa/46-kDa ratio was 0.1 for both the PCSK4-null and WT sperm. However, during WT sperm capacitation, it increased 6, 13, 20, and 39-fold after 30, 60, 90 and 120 min, respectively. In contrast, during PCSK4-null sperm capacitation, this ratio increased 7, 25, 50, and 74-fold after 30, 60, 90 and 120 min, respectively (Fig. 4B, upper panel). Capacitation did not alter the molecular size of ADAM3, but for equal amounts of sperm, the mutant samples appeared to contain less of this protein (Fig. 4B, lower panel).

Figure 4. *A representative gel showing the processing of proADAM2 and proADAM3 in mouse testis/sperm.* **A)** Testes from PCSK4-null and WT were homogenised in RIPA buffer containing complete protease inhibitor cocktail and sonicated. Homogenates were cleared by centrifugation, supernatants separated on SDS-PAGE, and immunoblotted for ADAM2 and ADAM3 (Top panel: ADAM2, Bottom panel: ADAM3). **B)** PGC washed sperm from PCSK4-null and WT were capacitated for 0 – 2 h and aliquots taken every 30 minutes. Samples were separated on SDS-PAGE and immunoblotted for ADAM2 and ADAM3 (Top panel: ADAM2, Bottom panel: ADAM3). [L/O represents PGC interphase sperm].


A
TESTIS
B
SPERM
kDa
180
130
100
70
55
45
35
25
IB: ADAM2
kDa
180
130
100
70
55
45
35
25
L/O
0
30
60
90
120
Capacitation Time (min)
IB: ADAM3

Conversion of 46-kDa ADAM2 to a 27-kDa Is Cholesterol Efflux-Dependent

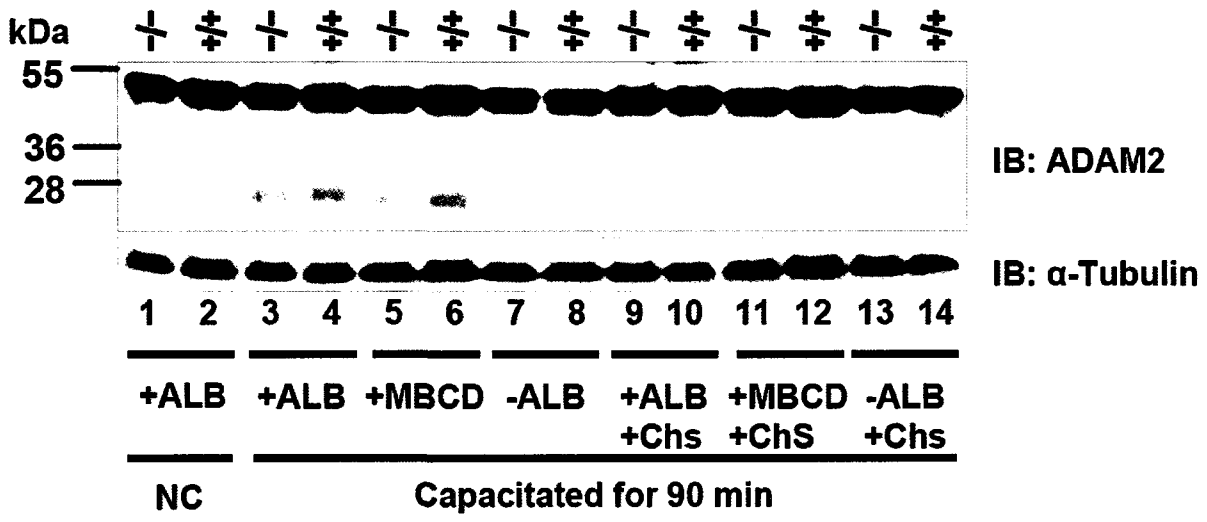
Cholesterol efflux is a major event during capacitation. *In vitro*, cholesterol efflux can be induced by cholesterol-extracting agents such as MBCD or albumin (33, 34). To determine if cholesterol efflux is a prerequisite for the ADAM2 conversion from the intermediate ~46-kDa form to the final ~27-kDa form, sperm from PCSK4-null and WT mice were capacitated for 0 and 90 min in (i) complete medium, (ii) complete medium saturated with ChS (35), (iii) albumin-free medium (KRB) (iv) KRB supplemented with ChS, (v) albumin-free medium supplemented with the MBCD, and (vi) albumin-free medium supplemented with MBCD and saturated with ChS (33). The level of ADAM2 immunoreactive forms was then determined by semi-quantitative immunoblotting using tubulin levels as an internal control. As shown in Fig. 5, the conversion was undetectable in non-capacitated sperm (lanes 1 and 2); it was observed in sperm capacitated in the presence of albumin (lanes 3 and 4) or MBCD (lanes 5 and 6), less so when albumin was omitted (lanes 7 and 8); and was significantly inhibited when ChS was supplemented in the presence of albumin (lanes 9 and 10) or MBCD (lanes 11 and 12 or in the absence of either cholesterol-extracting agent (lanes 13 and 14). These results suggest that changes in membrane dynamics associated with cholesterol efflux may either activate some ADAM2 convertase or expose susceptible sites to an already active convertase.

Is ADAM2 Processed by Kexin-Like Convertases?

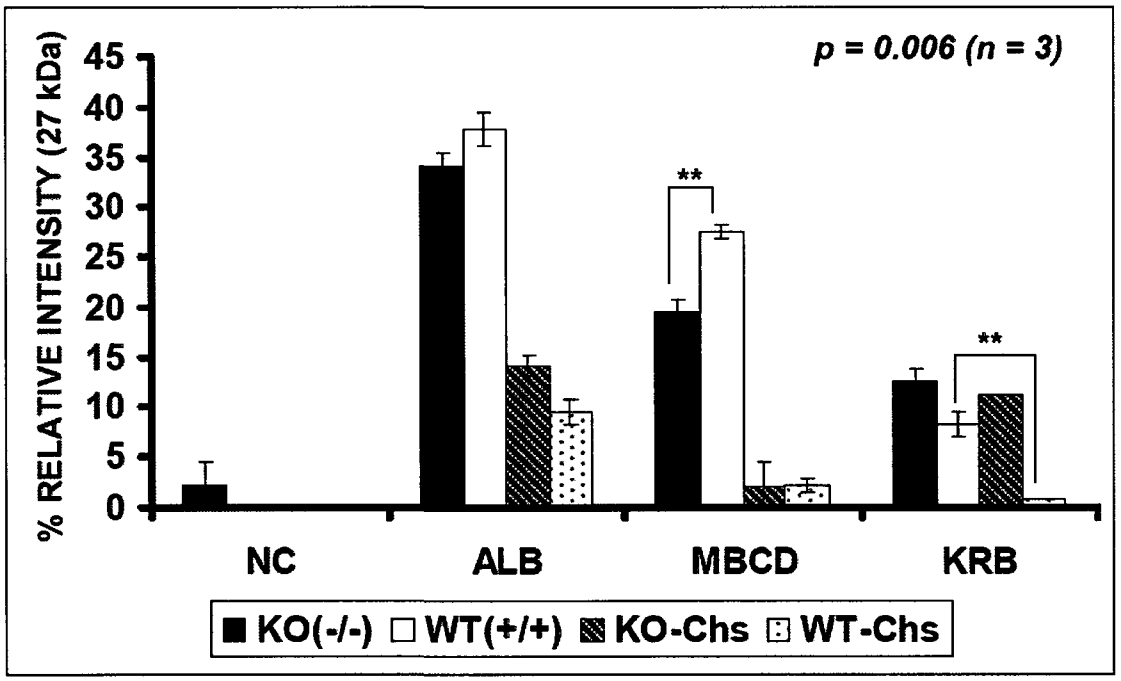
The fact that the ~46-kDa form of ADAM2 is processed to a ~27 kDa form in both PCSK4-null and WT sperm during capacitation indicates that PCSK4 is not required for this processing. It does not, however, exclude the possibility of this event being mediated by a related PCSK found in these cells, such as PCSK7. Because this observation is novel and

Figure 5. *Effect of Cholesterol efflux on the gradual conversion of ~46-kDa form of ADAM2 to ~27-kDa form.* **A)** A representative gel of PGC washed sperm from PCSK4-null and WT were capacitated for 90 minutes in different capacitation media: uncapacitated sperm samples (lanes 1 & 2); medium containing albumin (lanes 3 & 4); medium containing MBCD instead of albumin (lanes 5 & 6); medium without albumin nor MBCD (lanes 7 & 8); medium containing albumin and supplemented with ChS (lanes 9 & 10); medium containing MBCD instead of albumin and supplemented with ChS (lanes 11 & 12); medium supplemented with ChS without albumin or MBCD (lanes 13 & 14). Samples were separated on SDS-PAGE and immunoblotted for ADAM2. **B)** Graphical representation of the analysis of 3 separate experiments.

A

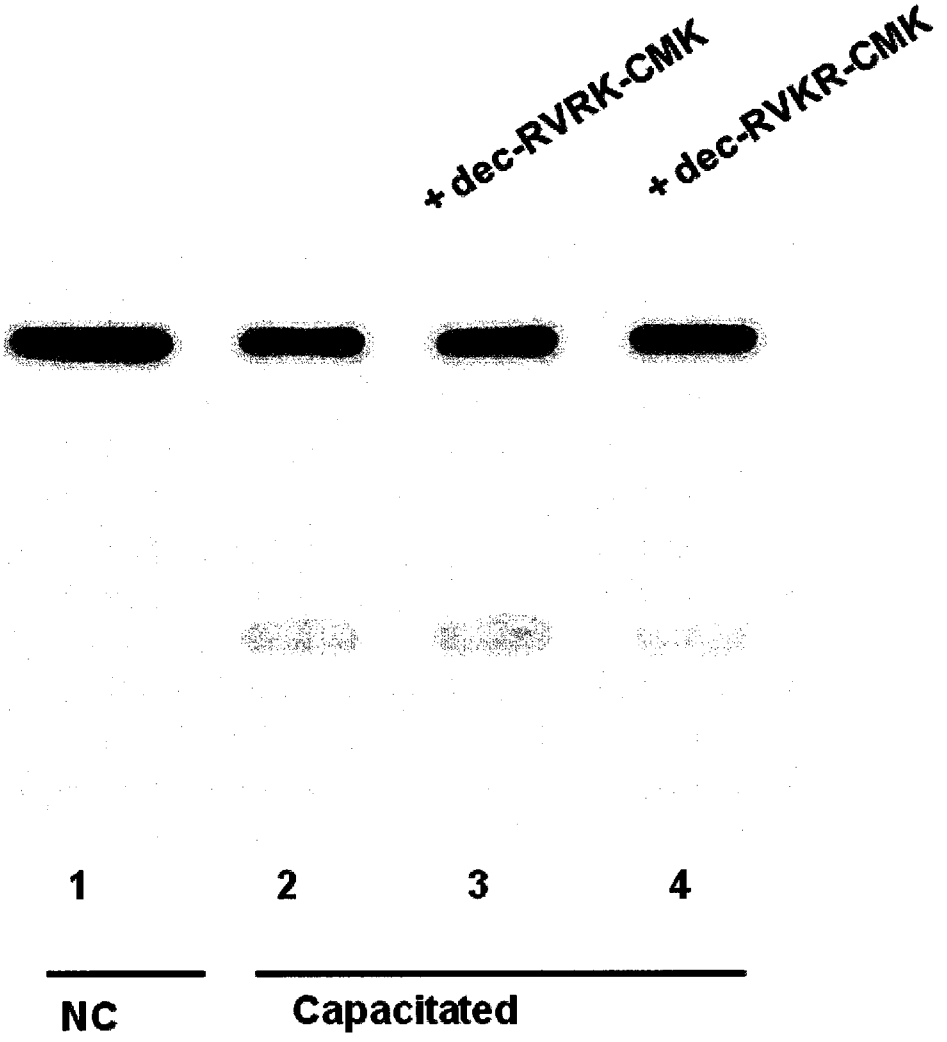

kDa
55
36
28
IB: ADAM2
IB: α-Tubulin
1 2 3 4 5 6 7 8 9 10 11 12 13 14
+ALB
+ALB
+MBCD
-ALB
+ALB +Chs
+MBCD +ChS
-ALB +Chs
NC
Capacitated for 90 min

B


p = 0.006 (n = 3)
% RELATIVE INTENSITY (27 kDa)
45
40
35
30
25
20
15
10
5
0
**
**
NC
ALB
MBCD
KRB
KO(-/-)
WT(+/+)
KO-Chs
WT-Chs

potentially interesting in relation to the redundancy of convertase activities in sperm, we decided to examine whether addition of dec-RVKR-CMK, the broad synthetic inhibitor of kexin-like convertases, could block this conversion during capacitation. The results in Fig. 6 show that dec-RVKR-CMK could do so (lane 4 versus 2) whereas the less specific dec-RVRK-CMK could not (lane 4 versus lane 3).

Figure 6. *Effect of general PCSK inhibitors on sperm proADAM2 processing.* PGC washed sperm from PCSK4 WT mouse were capacitated in complete medium for: 0 minute (lane 1), 90 minutes without any chloromethylketone (lane 2), 90 minutes with Dec-RVRK-CMK (lane 3), and 90 minutes with Dec-RVKR-CMK (lane 4). Sperm samples were separated on Tricine SDS-PAGE and immunoblotted for ADAM2.


+ dec-RVRK-CMK
+ dec-RVKR-CMK
1
2
3
4
NC
Capacitated

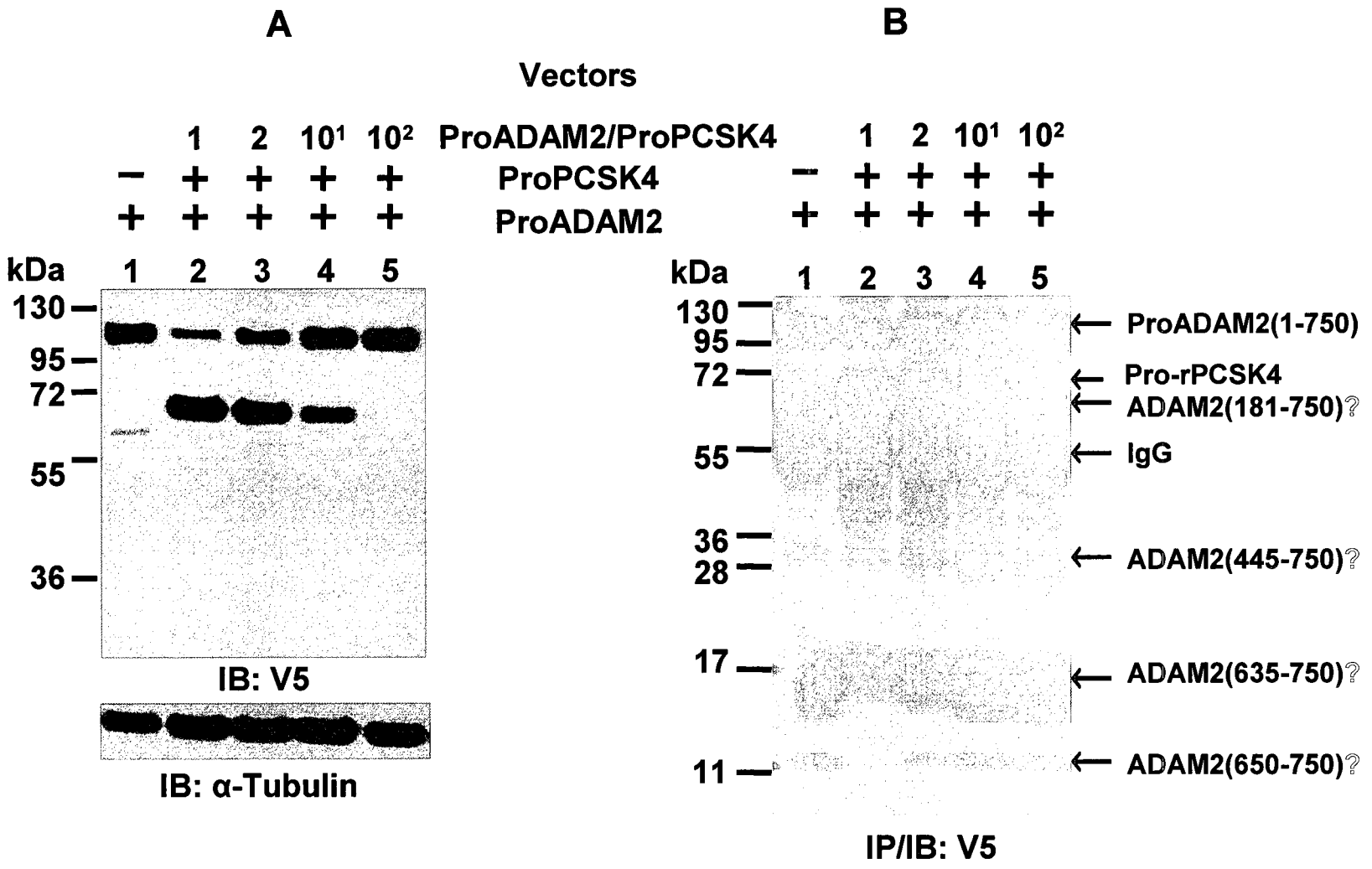
The primary sequence of proADAM2 contains several potential cleavage sites for kexin-like convertases (see Fig. 7). To verify if PCSK4 can process proADAM2, we generated plasmidic expression vectors for the expression of rat PCSK4 and mouse ADAM2, both with a C-terminal V5 tag. These vectors were transiently cotransfected in decreasing ADAM/PCSK4 ratios into QBI293A cells. Cell lysates were then analysed by immunoblotting with an anti-V5 antibody. As shown in Fig. 8A, cells transfected with the ADAM2 vector alone did not yield the ~46- or the ~27-kDa forms observed in sperm; instead, ~105- and ~66-kDa forms were observed (lane 1). Co-transfection with PCSK4 led to decreased levels of the ~105-kDa and the ~66-kDa bands in an enzyme concentration-dependent manner (lanes 2-5).

The cleavage products resulting from PCSK4 co-expression were not detectable with the V5 antibody. Assuming this to be due to their low amounts, we subjected larger volume of cell extracts to immunoprecipitation with the anti-V5 antibody to concentrate them and then analysed the precipitates by immunoblotting using the same antibody. In addition to the ~105 and ~66-kDa bands, additional bands were observed as the substrate/enzyme ratio was increased (Fig. 8B, lanes 2-5), including bands at ~29, ~17 and ~12 kDa that are consistent with cleavage at recognisable by kexin-like convertases. Loss of the V5 signal at a low substrate/enzyme ratio may be due to cleavage at the PCSK4-preferred $KSPR^{683-686}$ site, which released a ~6-kDa V5-tagged peptide that ran off the SDS-PAGE gel. On the other hand, the ~29-kDa protein may be generated by cleavage at another PCSK4-preferred $KLKR^{441-444}$ site, upstream of the disintegrin domain.

Figure 7. *Amino acid sequence of PreproADAM2-V5.* The amino-terminal signal peptide is given in green, the prodomain in blue, and the carboxyl-terminal V5 sequence in purple. Putative PCSK4 cleavage sites are shown in red, the disintegerin domain in underscored boldface; the transmembrane domain in underscored green. The integrin-binding motif QDECD is highlighted in blue and asparagine residues (N) susceptible to glycosylation in yellow.

1 20 20 30 40 50 60 70
MWLILLLLSGLSELGGLSQSQTEGTREKLHVQVTVPEKIRSVTSNGYETQVTYNLKIEGKTYTLDLMQKP
80 90 100 110 120 130 140
FLPPNFRVYSYDNAGIMRSLEQKFQNICYFQGYIEGYPNSMVIVSTCTGLRGFLQFGNVSYGIEPLESSS
150 160 170 180 190 200 210
GFEHVIYQVEPEKGGALLYAE**KDIDLR**DSQYKIRSI**KPQR**IVSHYLEIHIVVEKQMFEHIGADTAIVTQK
220 230 240 250 260 270 280
IFQLIGLANAIFAPFNLTVILSSLEFWMDENKILTTGDANKLLYRFLKWKQSYLVLRPHDMAFLLVYRNT
290 300 310 320 330 340 350
TDYVGATYQGKMCDKNYAGGVALHPKAVTLESLAIILVQLLSLSMGLAYDDVNKCQCGVPVCVMNPEAPH
360 370 380 390 400 410 420
SSGVRAFSNCSMEDFSKFITSQSSHCLQNQPRLQPSYK**MAVCGNGEVEEDEICDCGKKGCAEMPPPCCNP**
430 440 450 460 470 480 490
DTCKLSDGSECSSGICCNSCKLKRKGEVCRLAQDECDVTEYCNGTSEVCEDFFVQNGHPCDNRKWICING
500 510 520 530 540 550 560
TCQSGEQQCQDLFGIDAGFGSSECFWELNSKSDISGSCGISAGGYKECPPNDRMCGKIICKYQSENILKL
570 580 590 600 610 620 630
RSATVIYANISGHVCVSLEYPQGHNESQKMWVRDGTVCGSNKVCQNQKCVADTFLGYDCNLEKCNHHGVC
640 650 660 670 680 690 700
NN**KK**NCHCDPTYLPPDC**KRMK**DSYPGGSIDSGNKERAEPIPVRPYIASAYRS**KSPR**WPFFLIIPFYVVIL
710 720 730 740 750
VLIGMLVKVYSQRMKWRMDDFSSEEQFESESESKDTGKPIPNPLLGLDST

Figure 8. *Processing of mouse proADAM2 by PCSK4.* **A)** QBI293A cells were transfected with a pIRES vector for the expression of mouse proADAM2-V5 alone (lane 1) or together with a pCIneo vector for expression of FL-PCSK4-V5 in differing amounts (PCSK4, lanes 2 - 5). The final amount of plasmid was kept constant by adding a pCIneo empty vector. After 36 h, cell lysates were subjected to IB with mAb:V5. [1, 2, 10^1, and 10^2 represent ratios of the micrograms of ProADAM2 to that of ProPCSK4 co-transfected per each lane (lanes 2 – 5). Each lane (1 – 5) contained 1 μg of ProADAM2; lane 2 also contained 1 μg of ProPCSK4 giving a ratio of 1; lane 3, 0.5 μg of ProPCSK4 leading to a ratio of 2; lane 4, 0.1 μg of ProPCSK4 giving a ratio of 10; lane 5, 0.01 μg of ProPCSK4 resulting in a ratio of 100; rPCSK4-V5 bands are around 70 kDa]. The blot was stripped and re-probed with mAb:α-Tubulin to serve the internal control for equal loading. Processed intermediate, ~66-kDa form, increase as the amounts of PCSK4 decrease. **B)** QBI293A cells were transfected with a pIRES vector for expression of mouse proADAM2-V5 alone (lane 1) or together with a pCIneo vector for expression of FL-PCSK4-V5 in differing amounts (PCSK4, lanes 2-5). The final amount of plasmid was kept constant by adding a pCIneo empty vector. After 36 h, cell lysates were subjected to IP with mAb:V5 antibody and precipitates were analysed by IB with the same antibody. Possible processing intermediates (red?) increase as the amounts of FL-PCSK4-V5 decrease.


A
B
Vectors
1 2 10¹ 10² ProADAM2/ProPCSK4
− + + + + ProPCSK4
+ + + + + ProADAM2
kDa 1 2 3 4 5
130
95
72
55
36
IB: V5
IB: α-Tubulin
kDa 1 2 3 4 5
130
95
72
55
36
28
17
11
ProADAM2(1-750)
Pro-rPCSK4
ADAM2(181-750)?
IgG
ADAM2(445-750)?
ADAM2(635-750)?
ADAM2(650-750)?
IP/IB: V5

DISCUSSION

Despite their ability to move progressively, freshly ejaculated mammalian sperm are incompetent at fertilising a metaphase-II arrested egg. However, as they reside in the female genital tract for a finite period of time, they attain fertilisation competence by undergoing series of biochemical, morphological, and behavioural changes collectively termed as capacitation (14). Depending on the species, the physiological site of capacitation, *in vivo*, is either the oviduct or the uterus. Capacitation can however be stimulated to occur, *in vitro*, by mimicking the fluid content of the female reproductive tract (36). *In vitro* capacitation is accompanied by increased sperm metabolism (37, 38), changes in plasma membrane fluidity (39), changes in lectin reactivity (40, 41), hyperactivated motility of sperm (42), elevated intracellular pH (43), membrane hyperpolarisation (44), and increased tyrosine phosphorylation (45, 46). Activation of PKA-dependent signal transduction results in increased tyrosine phosphorylation of several sperm proteins, especially those in or associated with the sperm tail. It plays an important role in regulating sperm motility and acrosome reaction (14, 47). In seminal work by Visconti *et al.* (33, 34), it was shown that *in vitro* capacitation-induced tyrosine phosphorylation of sperm proteins was dependent on the presence of albumin, Ca^{2+}, and HCO_3^- ions in the capacitation medium. In our experiments, only HCO_3^- appears to be required for this modification. The reason for this discordance requires further investigation. One apparent difference is the use of PGC-purified sperm in our protocol and of washed sperm in theirs. Phosphorylation in the absence of added Ca^{2+} could be caused by the presence of trace amounts of the ions since no chelating agents were added to the medium.

The hyper-tyrosine phosphorylation of proteins for PCSK4-null sperm could be due to either enhanced tyrosine kinase activity or reduced tyrosine phosphatase activity (48). Lack of PCSK4 or its downstream product(s) may reduce the kinase/phosphatase ratio in sperm resulting in increased protein tyrosine phosphorylation during capacitation. This in turn will lead to increased responsiveness to ZP-induced signal transduction, and precocious acrosome reaction (9).

Tyrosine phosphatase activities are present in human and mouse sperm; these activities decline during capacitation (48). Whether any of these phosphatases require activation by PCSKs is presently unknown. It is interesting to note that there was no difference in basal tyrosine phosphorylation between PCSK4-null and WT sperm when capacitation was conducted in medium lacking albumin, Ca^{2+}, or in medium supplemented with the cAMP/PKA pathway inhibitor, H89. This basal phosphorylation would be insensitive to the negative regulatory effect of PCSK4 and products generated by its activity.

We used mass spectrometry to determine the identity of proteins migrating on SDS-polyacrylamide gel with a M*r* of ~70-kDa along with hyper-phosphorylated proteins. Essentially, the same proteins (see Table 1) were identified in PCSK4-null and WT sperm, whether capacitated or not. Some of these proteins are known to be phosphorylated and to play a role in sperm acquisition of fertilisation competence. AKAP3 and AKAP4 are both present in sperm tail and are involved in the PKA/cAMP signal transduction pathway leading to phosphorylation of proteins associated with the fibrous sheath (28, 49); AKAP4 presumably tethers PKA to fibrous sheath thereby facilitating phosphorylation of associated or neighbouring proteins such as, AKAP3 or glyceraldehyde-3-phosphate dehydrogenase-S (GAPDS) that regulate flagellar activity (29); ODF protein, a ~70-kDa protein, is a major

cytoskeletal component of sperm tail (50) and may be involved in hyperactivated motility; dihydrolipoamide S-acetyltransferase, a component of pyruvate dehydrogenase S complex, is reported to be involved in sperm hyperactivation, tyrosine phosphorylation, capacitation, and acrosome reaction (30, 51). One trait of the PCSK4-null sperm is reduced hypermotility following capacitation (13). This indicates that, in spite of its final localisation on the membrane overlying the acrosome, PCSK4 may influence physiological events that are manifested in the sperm tail. We cannot, at this time, confirm which of these protein(s) is/are specifically hyper-phosphorylated in the PCSK4-null sperm during *in vitro* capacitation.

Among the potential proprotein convertase substrates in gonadal cells, the only one proven to be a PCSK4 physiological substrate is pro-pituitary adenylate cyclase-activating protein (proPACAP) (52). It is not processed into its active peptides in both the testes and ovaries of PCSK4-null mice (52). PACAP peptides are reportedly found in sperm too (53), but their levels in PCSK4-null and WT sperm have never been compared. On the other hand, our study on the intracellular levels of cAMP did not reveal any significant difference between the two types of sperm (our unpublished results).

ADAM2 and ADAM3 were proteins of interest in our effort to unravel the molecular events underlying the infertility phenotype of PCSK4-null mice. Like PCSK4, they are primarily expressed in testicular germ cells and are found on the plasma membrane overlying the acrosome. Like PCSK4-null male mice, male ADAM2 and ADAM3-null mice are infertile partly due to inefficient binding to egg ZP. Like all ADAMs, they are biosynthesised as multidomain type-1 transmembrane preproproteins made up of a signal peptide, a prodomain, a metalloproteinase domain, a disintegrin domain, a cysteine-rich domain, an epidermal growth factor (EGF)-like domain, a transmembrane domain, and a cytoplasmic tail

(54, 55). These proproteins undergo successive proteolytic cleavages, removing the prodomain and the metalloproteinase domain, leaving the disintegrin domain N-terminally exposed (54). The primary sequences of both proADAMs carry consensus cleavage motifs for kexin-like PCSKs around their presumed processing sites, raising the possibility that they may be processed by this subfamily of convertases, including PCSK4. The molecular forms of processed proteins in the testicular and sperm extracts of PCSK4-null and WT mice were very similar, suggesting that either PCSK4 is not involved or is not the only PCSK involved in the proteolytic maturation of these precursors. However, for comparable number of sperm, the post-capacitation levels of the processed products in PCSK4-null sperm were less abundant than in WT, suggesting a differential loss.

Our data show that, in line with previous reports (18, 32), testicular proADAM2 is not efficiently processed whereas nearly half of proADAM3 is converted to a ~50-kDa form. There was no significant difference in the level of processed products between PCSK4-null and WT mice, suggesting that PCSK4 is not required for this modification. In epididymal sperm from both genotypes, proADAM2 and proADAM3 were completely converted to fragments of ~46-kDa and ~50-kDa respectively. Capacitation induced a gradual conversion of the ~46-kDa ADAM2 to a ~27-kDa form which appeared to be mediated by a PCSK-like enzyme, as it could be blocked by the PCSK-specific inhibitor dec-RVKR-CMK. This substrate-enzyme interaction may be facilitated by changes in membrane fluidity associated with cholesterol efflux during capacitation since it was reduced when the cholesterol acceptor, albumin or MBCD, was omitted from the capacitation medium or when cholesterol was supplemented to the medium. In the presence of MBCD, the conversion was more inhibited in PCSK4-null than in WT sperm, suggesting that some level of spontaneous cholesterol efflux

occurs in the WT but not in the PCSK4-null sperm under the capacitation conditions. Whether lack of PCSK4 makes sperm relatively cholesterol deficient is unknown. It is possible PCSK4 lack affects the nature and the extent of changes in sperm lipid and protein composition that are induced by capacitation, leading to both enhanced acrosome reactivity and diminished binding to egg ZP. Such changes in proteins have been reported in null mice for ADAM2, ADAM3, and testicular calmegin (17, 56, 57).

The inhibitory effect of dec-RVKR-CMK on capacitation-associated processing of the ~46-kDa ADAM2 led us to examine whether PCSK4, as a surrogate kexin-like PCSKs, could process proADAM2 when co-transduced into cultured cells. At a substrate/enzyme ratio of 100, proADAM2 was processed into fragments of about 65, 28, and 17 kDa that could be interpreted to derive from cleavages after the $KPQR^{177-180}$, $KLKR^{441-444}$, and $KR^{648-649}$ respectively. Without its terminal V5 tag, ~28-kDa would migrate as a ~27-kDa protein, like the ADAM2 processed form seen in capacitated sperm. The $KLKR^{441-444}$ motif is located 8 amino acids upstream of disintegrin motif (QDECD). Processing at this site may expose this motif allowing its interaction with egg integrins.

While these *ex vivo* data suggest that PCSK4 may process proADAM2, the study of its processing in PCSK4-null clearly indicate that it is not required for this processing. PCSK7, which is known to be expressed in testicular germ cells (58) and is found in epididymal sperm (our unpublished data), might mediate this conversion in PCSK4-null sperm. However, PCSK7-null mice are fertile (59), suggesting that, even if it activates some substrates in common with PCSK4, PCSK7 cannot substitute for all PCSK4 functions in male fertility.

In summary, we have shown that lack of PCSK4 is associated with quantitative changes in the phosphorylation and proteolysis of sperm proteins during capacitation. More investigation is needed to determine how and to what extent these changes might contribute to the loss of fertilising ability by PCSK4-null sperm.

ACKNOWLEDMENTS

The authors thank Ms. Adrianna Gambarotta for her help in animal maintenance, Dr. N.G. Seidah of IRCM, Montreal, QC for providing us with the pIRES-hNARC1-V5-EGFP plasmid, and Dr. George L. Gerton, University of Pennsylvania, Philadelphia, PA for providing us with the Anti-AKAP4 antibody. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

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IV. MANUSCRIPT III: ProPCSK4 is slightly matured to its active form in transfected somatic cells: evidence of interactions with BiP and of enzymatic activity

Charles GYAMERA-ACHEAMPONG, Francine SIROIS, Nicholas J. DENIS, Daniel FIGEYS, and Majambu MBIKAY. **ProPCSK4 is slightly matured to its active form in transfected somatic cells: evidence of interactions with BiP and of enzymatic activity.** *Re-submitted to Biochimie.*

The manuscript was written in close collaboration with Dr. Majambu Mbikay.

Different extraction methods were used to investigate the sperm surface localisation of mPCSK4. Through conventional cloning techniques, transfections, pulse-chase analyses, and indirect immunofluoscence; the nature of PCSK4 association to membranes, as well as the biosynthesis, maturation, and its enzymatic activity were studied.

CONTRIBUTIONS OF AUTHORS

The LC-MS/MS analysis (Table 1) was performed by Mr. Nicholas Denis; Ms. Francine Sirois contributed to Figures 1, 2, and 3. I performed to the sample preparation, SDS-PAGE, and the silver staining leading to the LC-MS/MS analysis. I also contributed to all the remaining aspects of this manuscript (tools generation, technical aspects, experimental aspects, etc.). Dr. Daniel Figeys provided an intellectual contribution to the LC-MS/MS analysis presented herein, and he also critically reviewed the manuscript during its preparation.

Re-submitted to Biochimie on April 30, 2009.

ProPCSK4 is slighlty matured to its active form in transfected somatic cells: evidence of interactions with BiP and of enzymatic activity

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Short Title

Biosynthesis, maturation, and activation of PCSK4

Re-submitted to Biochimie on April 30, 2009

ABBREVIATIONS

BiP, immunoglobulin heavy-chain binding protein; CPIC, complete protease inhibitor cocktail; DAPI, 4'-6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's Medium; ER, endoplasmic reticulum; GRP, glucose-regulated protein; HEK, human embryonic kidney; HRP, horseradish peroxidase; hPCIN, human Protein C inhibitor; IGF, Insulin-like Growth Factor; IP/IB, immunoprecipitation/immunoblotting; LC-MS/MS, liquid chromatography tandem mass spectrometry; mAb, monoclonal antibody; MCS, multiple cloning site; NARC-1, neural apoptosis regulated convertase-1; ORF, open reading frame; pAb, polyclonal antibody; PBS, phosphate buffered saline; PC, proprotein convertase; PCSK, proprotein convertase subtilisin/kexin type; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SKI-1/SIP, Subtilisin/Kexin Isozyme-1/Site 1 Protease.

Abstract

Proprotein Convertase Subtilisin/Kexin Type 4 (PCSK4), also known as Proprotein Convertase 4 (PC4), is a serine endoproteinase primarily expressed in testicular germ cells and in sperm. Inactivation of its gene in mouse causes male infertility. From studies of the biosynthesis of PCSK3/furin, its closest relative, it has been inferred that PCSK4 is synthesised in the endoplasmic reticulum as a zymogen; that it is rapidly matured by autocatalytic cleavage between the prodomain and the catalytic domain; that the cleaved prodomain remains attached to the mature enzyme, and that the enzyme is finally activated by the removal of the prodomain peptides following a secondary cleavage within the prodomain. In this study, we used human embryonic kidney 293 (HEK293) cells to study the biosynthesis of transduced rat or human PCSK4. Our results show that the bulk of PCSK4 remains as an intracellular zymogen, presumably trapped in the endoplasmic reticulum where it interacts with the general molecular chaperone Glucose-regulated protein 78/Immunoglobulin heavy-chain Binding Protein (GRP78/BiP). PCSK4 activity was detectable nonetheless, as suggested by increased processing of co-expressed pro-insulin growth factor 2. These data suggest that, unlike testicular germ cells, somatic cells lack the intracellular environment and the interacting molecules necessary for efficient maturation and trafficking of proPCSK4.

Keywords: proprotein convertase, prodomain, chaperone, glucose-regulated protein 78, zymogen, trafficking.

1. Introduction

Proprotein Convertase Subtilisin/Kexin Type 4 (PCSK4), also known as Proprotein Convertase 4 (PC4), belongs to a 9-member family of calcium-dependent serine endoproteinases structurally related to bacterial Subtilisin and to yeast Kexin. Other family members are: PCSK1, PCSK2, PCSK3, PCSK5, PCSK6, PCSK7, PCSK8, and PCSK9 also known respectively as PC1/3, PC2, Furin, PC5/6, PACE4, PC7/8, Subtilisin Kexin Isozyme-1/Site 1 Protease (SKI-1/SIP), and Neural Apoptosis Regulated Convertase-1 (NARC-1). PCSK1 to 7 belong to the Kexin subfamily; PCSK8 and PCSK9 to the pyrolysin and proteinase K subfamilies, respectively.

These enzymes are expressed in nearly all nucleated cells in different combinations. They are biosynthesised as secretory precursor proteins made of several successive domains: a signal peptide (SP), a prodomain, a catalytic domain, a P(rotease) domain, and a carboxyl terminal (CT) domain. The SP channels the nascent polypeptide into the secretory pathway and is removed during translation by a signal peptidase. The prodomain serves as an intramolecular chaperone and an inhibitor; its removal renders the mature enzyme fully active. The catalytic domain contains the His, Asp, Ser triad typical of serine proteases; the P domain influences calcium and pH-dependence, besides contributing to the stability of the enzyme. The CT domain, the most variable among PCSK family members, mediates protein-protein interactions; for some of the members, it integrates the enzyme to the membrane lipid bilayers via a transmembrane (TM) domain (reviewed in [1-3]).

PCSK1 to 7 cleave their substrates on the carboxyl side of basic residue (Arg or Lys), most often when this P1 (position 1) is preceded by another basic residue at P2, P4, P6, or P8 [1]. PCSK8 and PCSK9 cleave after non-basic residues [4]. Collectively, these enzymes are

responsible for the proteolytic activation of a variety of precursor proteins in the secretory pathway. Functional products of this modification include precursors of hormones, neuropeptides, growth factors, blood coagulation factors, plasma proteins, receptors, extracellular matrix (ECM) proteins, transcription factors, viral glycoproteins, and bacteria toxins [1, 5].

PCSK4 is encoded by a 9.5-kb, 15-exon gene [6]. Its gene is located on mouse chromosome 10 (locus symbol, *Pcsk4*) and on human chromosome 19 (locus symbol, *PCSK4*) [7]. In mouse, this gene is transcribed to a 2.8-kb mRNA and 5 alternatively spliced isoforms [6, 8, 9]. These transcripts are most abundant in spermatocytes and in round spermatids [9, 10]. The protein is found in these same germ cells, but persists and accumulates in elongated spermatids and in sperm. In the latter cells, it is found on the plasma membrane overlying the acrosome [11]. *Pcsk4* transcripts have also been detected in mouse ovaries [12], frog ovaries and testes [13], as well as in human placenta [14].

The primary localisation of PCSK4 in testicular germ cells and in sperm suggested that this enzyme may be important for male fertility. Indeed, knockout (KO) of the *Pcsk4* gene in mouse causes male infertility [15]. These sperm exhibit maximal acrosome reaction at concentrations of zona pellucida (ZP) that are ineffective on WT sperm, are less efficient at binding to egg ZP, and are incapable of fertilising eggs *in vitro* [11]. Females KO mice are subfertile [15], probably due to a reduced response to gonadotrophins leading to impaired folliculogenesis, ovulation, and luteinisation [12].

Although there are several potential PCSK4 substrates in gonadal cells, the only proven one is pro-pituitary adenylate cyclase-activating protein (proPACAP). Indeed, in both the testes and ovaries of *Pcsk4* KO mice, the precursor is not processed into its active peptides, PACAP27 and PACAP38 [16].

In spite of the physiological importance of PCSK4, little is known about its biosynthesis, maturation, and transport. Based on its cDNA sequence, mouse mature PCSK4 is predicted to be a soluble protein of ~62-kDa; yet in mouse sperm, it is found at the cell surface and migrates on SDS-polyacrylamide gel as ~54 kDa protein. This work was initiated to elucidate the molecular modifications proPCSK4 undergo when transduced into human embryonic kidney HEK293 cells. Our results indicate that, in these cells, the zymogen is slightly converted to its mature form and is retained in the ER in association with Glucose-regulated protein 78/Immunoglobulin heavy-chain Binding Protein (GRP78/BiP), suggesting that efficient maturation and transport of PCSK4 in testicular germ cells is facilitated by intracellular conditions or interacting proteins not found in somatic cells.

2.1. *Animals*

Mice used in this study were male C57BL/6J (B6) WT or B6-*Pcsk4*^{tm1Mbi} congenics (*Pcsk4* KO) [15]. They were housed, fed, and treated according to the Guidelines of the Canadian Council on Animal Care under a protocol approved by the institutional Animal Care Committee.

2.2. *Antibodies and other materials*

Three rabbit anti-PCSK4 (α-PCSK4) polyclonal antibodies (pAb) were used in this study: (i) α-mPCSK4 pAb, previously described as the Belgian pAb [11] raised against a recombinant fragment of mouse PCSK4 [mPCSK4, amino acids (aa) 133-506] covering most of the catalytic and the P domain; (ii) α-rPCSK4-606 raised against full-length rat PCSK4 (rPCSK4) [11]; (iii) α-rPCSK4NT raised against a recombinant rPCSK4 peptide (aa 27-133) covering all the prodomain (aa 27-110) and the first 23 residues of the catalytic domain. The properties of these pAb are described in Supplemental Table 1. The following mouse monoclonal antibodies (mAb) and pAb, as well as reagents and kits were purchased from commercial sources: α-V5 mAb (Invitrogen, Burlington, ON); α-Flag M2 mAb (Sigma, St. Louis, MO); α-α-tubulin mAb (Sigma); α-GRP78/BiP antibody pAb, (Abcam, Cambridge, MA); α-hSerpin A5 mAb (R & D Systems, Minneapolis, MN); horseradish peroxidase (HRP)-conjugated sheep α-mouse IgG (Amersham, Buckinghamshire, UK); HRP-conjugated donkey α-rabbit IgG (Amersham); Alexa fluor 594 goat α-rabbit IgG (Molecular Probes, Eugene, OR); Western Lightning Chemiluminescence Reagent Plus Kit used for

immunoblotting (IB) (Perkin-Elmer, Boston, MA); Protein A-agarose (Sigma); Geneticin (G418, Invitrogen); Complete protease inhibitor cocktail (CPIC. Roche Diagnostics, Mannheim, Germany).

2.3. Expression vectors

Expression vectors were generated by cloning an open reading frame (ORF) of the cDNAs of interest into the multiple-cloning site (MCS) of selected eukaryotic expression vectors using standard molecular biology techniques (see Supplemental Materials and Methods). **(A)** For expression of rPCSK4, the corresponding full and 3′-truncated ORFs were amplified by PCR from a previously cloned cDNA [9]. They were inserted into the pCIneo vector (Promega, Madison, WI) in frame with a synthetic double stranded oligonucleotide encoding the simian virus 5 (V5) epitope, thus generating the pCIneo-rPCSK4-V5 and pCIneo-rPCSK4ΔCT-V5 vectors, respectively. **(B)** For expression of human PCSK4 (hPCSK4), the cognate cDNA representing the full ORF (aa 1-755) was amplified by RT-PCR from total RNA of HEK293 cells and inserted into the MCS of the pCIneo plasmid, generating the pCIneo-hPCSK4. Two variants of this vector, both C-terminally tagged with the hexahistidinyl epitope (H6): pCIneo-prepro-hPCSK4ΔTM-H6 and pCIneo-prepro-hPCSK4ΔDoP-H6 (DoP stands for downstream of P domain) were generated to express hPCSK4 terminating before the TM domain (aa 1-705) and after the P domain (aa 1-551), respectively. A third variant, hPCSK4ΔPro, encoding the pre domain directly fused to the catalytic domain was also generated. In addition, restriction fragments or amplicons from this cDNA representing segments of the ORF were cloned into the MCS of the pIRES-EGFP vector (Invitrogen): the pphPCSK4-V5 ORF encoding the prepro domain of hPCSK4 with a

carboxyl V5 epitope. **(C)** For expression of the serpin, human Protein C inhibitor (hPCIN), the corresponding cDNA was amplified from human testicular cDNA and inserted into the MCS of the pCIneo plasmid. The accuracy of all each ORF was ascertained by DNA sequencing. **(D)** The expression vector pro-IGF2 was a gift from Dr. Stephen J. Duguay (Genzyme Corp, Cambridge, MA).

2.4. Site-directed mutagenesis

Mutagenesis was performed using the QuickChange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and appropriate oligonucleotide primers (see Supplemental Materials and Methods). The following mutations were introduced: $S^{373}A$, converting the active site Ser of hPCSK4 to an Ala; GTIFTFRSA$^{283\text{-}291}$TPAKSERDV converting sequence around the scissile bond (P1 underlined) in the reactive site loop (RSL) of hPCIN with one around the PCSK4-susceptible site in the sequence of human pro-insulin-like-growth factor 2 (proIGF2) [17].

2.5. Cell culture and transfection

For transfection, HEK293 cells were seeded at 8 x 10^5 cells/3.5-cm dish in 2.5 ml of Dulbecco's Modified Eagle's Medium (DMEM) with 10% foetal bovine serum (FBS) (Invitrogen) at 37°C under 5% CO_2. At about 90% confluence, cells were transfected with a total of 2 μg of plasmid DNA of pCIneo (empty) and/or the appropriate expression vector using the Lipofectamine Reagent as instructed by the manufacturer (Invitrogen). Thirty-six

hours after transfection, cells extracts were prepared for IB or IP/IB. Alternatively, the cells were incubated for at least 2 weeks in medium containing 0.6 mg/ml of G418 to select the stable transfectants: HEK293(rPCSK4-V5) and HEK293(rPCSK4ΔCT-V5).

2.6. Indirect immunofluorescence

HEK293(rPCSK4-V5) cells were first seeded into Lab-Tek II Chamber Slide System and allowed to grow for 18 h. They were then subjected to the following successive treatments, with 3 in-between washes with PBS (0.02 M sodium phosphate buffer with 0.15 M sodium chloride, pH 7.4): they were (i) fixed with 4% paraformaldehyde for 2 h; (ii) permeabilised with 0.5% TX-100 in PBS for 2 h; (iii) incubated for 1 h at room temperature in PBS containing 20% normal horse serum (NHS) to saturate non-specific antibody-binding sites; (iv) with α-rPCSK4-606 pAb (1:200 dilution) for 45 min; (v) with Alexa fluor 594 goat α-rabbit IgG (1:150 dilution) for 1 h. After applying drops of Vectashield Mounting Medium with DAPI, slides were examined under a Zeiss Axioplan epifluorescence microscope (Carl Zeiss Canada, Mississauga, ON) at 500x magnification.

2.7. Metabolic labelling and pulse-chase

Cells were incubated in serum-free medium for 30 min to reduce the intracellular content of methionine-cysteine. The serum-free medium was substituted with medium containing 750 μCi ^{35}S-Met-Cys/ml and incubation resumed for 15 min of (^{35}S) metabolic pulse-labelling. Radioactive media were replaced with fresh, non-radioactive, complete

medium and incubation was continued for varying lengths of time (15-240 min; chase). Spent media and cell lysates were collected at specific time intervals and frozen at -80°C until analysed.

2.8. Differential extraction of mouse sperm proteins

To collect sperm, wildtype (WT) and *Pcsk4* KO mice were sacrificed under anaesthesia by cervical dislocation; cauda epididymal and vas deferens sperm from both genotypes were collected into PBS. After sedimentation, sperm proteins were differentially solubilised at 4°C as described by Shetty *et al.* [18]: (i) in Celis buffer (9.6 M urea, 2% Nonidet P-40, 100 mM DTT) for 45 min; (ii) in PBS containing 1% Triton X (TX)-100 for 30 min; (iii) in PBS containing 1 M NaCl for 30 min. All solubilisation buffers contained complete protease inhibitor cocktail (CPIC) as recommended by the manufacturer (Roche Diagnostics). Particulate materials were pelleted by centrifugation at 10 000 x g for 4 min; pellets and supernatants were collected. For phase partition of proteins, sperm were incubated in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) containing 1.7% TX-114 for 60 min. After centrifuging at 10 000 x g for 20 min to pellet particulate materials, supernatants were adjusted to 1% TX-114, warmed to 30°C for 3 min, and separated into detergent and aqueous phases by a 3-min centrifugation at 10 000 x g. Proteins in pellets, supernatants, and TX-114 partition phases were analysed for PCSK4 by IB.

2.9. Immunoprecipitation and immunoblotting

Unless stated otherwise, cells were homogenised and sonicated in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing CPIC. Homogenates were centrifuged at 14 000 x g for 30 min at 4°C and supernatants collected. Protein concentrations in these extracts were determined by the Bradford dye-binding method [19] using reagents and protocol from Bio-Rad Laboratories (Mississauga, ON). IP and IB were conducted as we have previously described [20].

2.10. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis)

SDS-PAGE silver stained gel bands were excised and subjected to in-gel tryptic digestion as previously described [21]. Digestions were carried out for 16 h using sequencing grade trypsin from Promega (Madison, WI). Peptides from gel bands were extracted and reconstituted in 30 µl of 5% formic acid (FA). Peptides were then analysed by LC-MS/MS by loading them at 5 µl/min onto a 200 µm x 50 mm precolumn packed with 5 µm YMC ODS-A C18 beads (Waters, Milford, MA) using an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA). Following a desalting step, the flow was split and peptides were eluted through a second 75 µm x 50 mm column packed with the same beads, at approximately 0.2 µl/min using a 5 – 80% gradient of acetonitrile with 0.1% FA (J. T. Baker, Northbrook, IL) for 1 hr. The LC effluent was electrosprayed into an LTQ linear ion-

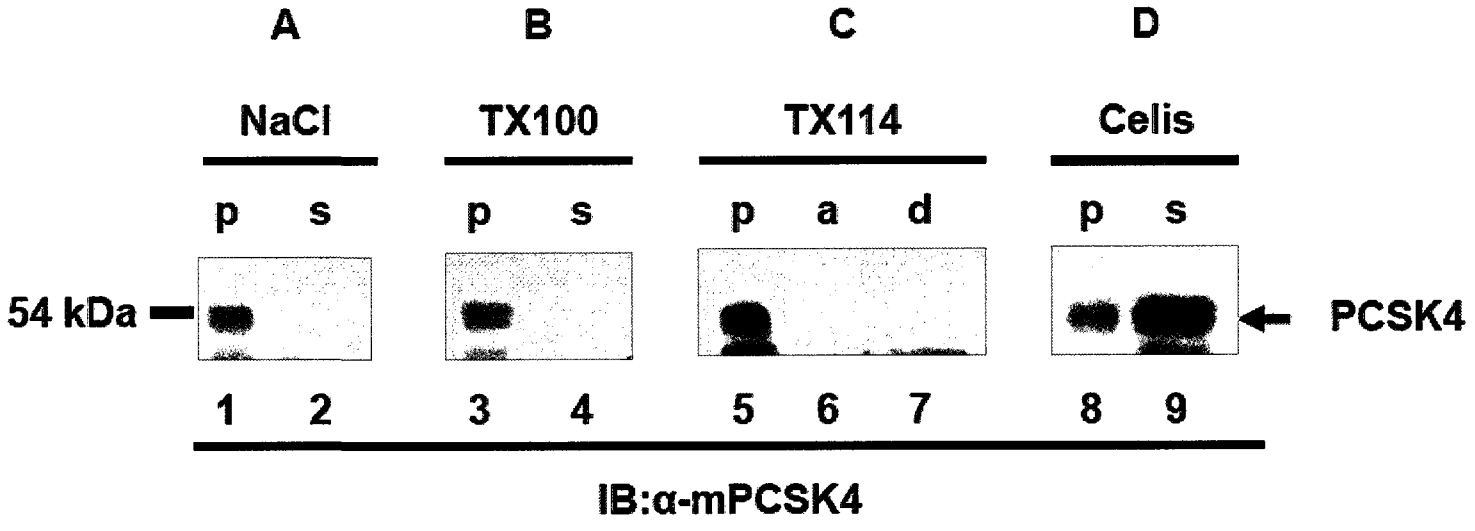
trap mass spectrometer (Thermo-Electron, Gormley, ON), and MS/MS spectra were acquired in a data-dependant acquisition mode that automatically selected and fragmented the four most intense peaks from each MS spectrum generated. Peptide and MS/MS mass tolerances were set at 0.2 and 0.8 Da, respectively. MS/MS data were then analysed and matched to protein sequences in the NCBI database (nrdb) using the MASCOT database search engine (Matrix Science, London, UK) with carbamidomethyl as a fixed modification and oxidation as a variable modification.

3. Results

3.1. Mouse PCSK4 is tightly associated with membranes

We have previously reported the localisation of PCSK4 on mouse sperm surface [11]. Mouse PCSK4 lacks a recognisable transmembrane domain. In an effort to explain its cell surface location, we extracted epididymal sperm proteins in buffers containing ingredients that should: (A) disrupt ionic interactions (1 M NaCl); (B) disperse lipid bilayers (1% TX-100); (C) partition extracted proteins into aqueous-insoluble or detergent-soluble phases (1.7% TX-114); (D) break hydrogen and disulfide bonds (100 mM DTT). Extracts were fractionated by centrifugation into supernatants and pellets, or for TX-114 treated samples, into aqueous and detergent phases. These fractions were analysed by IB using the α-mPCSK4 pAb. The expected ~54-kDa PCSK4-specific band should be observed in WT samples only [11]; this band was present in the pellet fractions when proteins were extracted in the presence of 1 M NaCl (Fig. 1A, lane 1), 1% TX-100 (Fig. 1B, lane 5), or 1.7% TX-114 (Fig. 1C, lane 9). Only Celis buffer was able to release a substantial amount of PCSK4 into the soluble fraction (Fig. 1D, lane 16). When the various components of the Celis buffer were tested, 9.6 M urea was the only component able to render sperm PCSK4 soluble (not shown). These results suggest that PCSK4 is tightly bound to water-insoluble and detergent-soluble components of the plasma membrane overlying the acrosome through hydrophobic interactions.

Figure 1. *Mouse PCSK4 is tightly associated with membranes.* Epididymal sperm from WT were solubilised in (A) 1 M NaCl. (B) 1% TX-100. (C) 1.7% TX-114. (D) Celis Buffer. Extracts were fractionated by centrifugation into supernatants and pellets, or for TX-114 treated samples, into aqueous and detergent phases. Fractions were resolved by 8.5% Tricine SDS-PAGE and analysed by immunoblotting using the anti-mPCSK4 antibody.

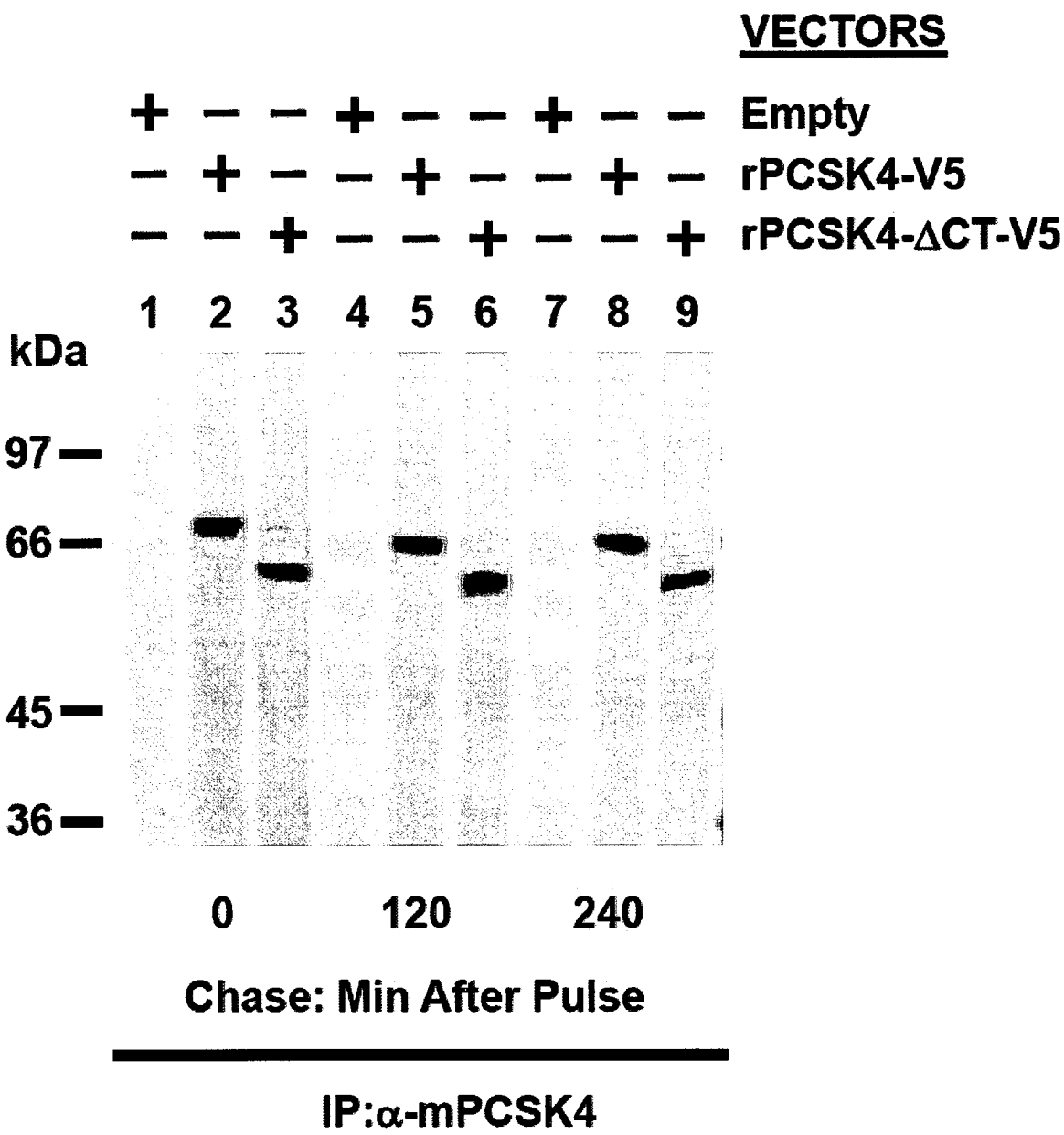


a, aqueous phase d, detergent phase p, pellet s, supernatant

3.2. Rat PCSK4 is associated with intracellular membrane fraction

We suspected that the interaction of PCSK4 with membranes might be mediated by a conserved 20-aa long and Tyr-rich hydrophobic region located in the CT domain ($GYYFNTGTLYYYTLLLYGTA^{559-578}$). To investigate this possibility, we constructed the plasmid vectors pCIneo-rPCSK4-V5 and pCIneo-rPCSK4ΔCT-V5 for expression of full-length $rPCSK4^{1-654}$ and truncated $rPCSK4^{1-527}$, respectively, both carrying a C-terminal V5 tag. These vectors were transfected into HEK293 cells; HEK293(rPCSK4-V5) and HEK293(rPCSK4ΔCT-V5) stable lines were established. The transfected cells were tested for their ability to express and mature these proteins in a pulse-chase experiment. After a 15-min pulse labelling with radioactive ^{35}S-Met-Cys, two major radioactive bands of ~72 kDa and ~56 kDa were immunoprecipitated by the α-mPCSK4 antibody from lysates of HEK293(rPCSK4-V5) (Fig. 2, lane 2) and HEK293(rPCSK4ΔCT-V5) (Fig. 2, lane 3) cells, respectively. These bands presumably represented zymogen forms of rPCSK4. To our surprise, after a chase of 2 h and 4 h, these bands remained largely unchanged (Fig. 2, lanes 5, 6, 8 and 9), i.e. were not converted to smaller forms of predicted molecular weights of 62 kDa for rPCSK4-V5 and 46 kDa for rPCSK4ΔCT-V5, representing products of zymogen maturation by autocatalytic removal of the prodomain. Neither the zymogens nor the mature forms were detected in the medium (not shown).

Figure 2. *Rat PCSK4 is associated with intracellular membrane fraction.* Plasmid vectors: pCIneo-rPCSK4FL-V5 and pCIneo-rPCSK4ΔCT-V5 for expression of full-length rPCSK4$^{1-654}$ and truncated rPCSK4$^{1-527}$, respectively, both carrying a C-terminal V5 tag were constructed. Transfections into HEK293 cells were carried out, and stable lines established. The ability of the cells to express and mature the expected proteins was assessed by a 15-min pulse labelling with radioactive ^{35}S-Met-Cys followed by in a 4-h chase. Labelled proteins were immunoprecipitated with anti-mPCSK4 antibody, resolved by 8.5% Tricine SDS-PAGE, and detected by phosphorimaging.


VECTORS
+ − − + − − + − − Empty
− + − − + − − + − rPCSK4-V5
− − + − − + − − + rPCSK4-ΔCT-V5
1 2 3 4 5 6 7 8 9
kDa
97
66
45
36
0
120
240
Chase: Min After Pulse
IP:α-mPCSK4

To examine the intracellular compartmentalisation of rPCSK4, HEK293(rPCSK4-V5) cells were grown at either 37°C or 25°C to attenuate protein expression. Lysates were fractionated into Nuclear/Mitochondrial pellet (P15), ER/Golgi/Microsomal pellet (P100), and soluble fraction (S100) by differential centrifugation. These fractions were analysed by IB using the α-V5 antibody. The largest amount of rPCSK4-related proteins was found in the P15 fraction (Fig. 3A, lanes 1 and 2); the P100 fraction contained less (Fig. 3A, lanes 5 and 6) and the S100 fraction the least (Fig. 3A, lanes 9 and 10). Interestingly, the two pellet fractions contained, besides what appear to be degradation products, a relatively intense band of ~58-kDa that may be a product of a specific cleavage. Growing the cells at 25°C made this band more obvious as the lower temperature significantly reduced pro-rPCSK4 degradation (Fig. 3A, lanes 2 and 6). These results suggest that, after biosynthesis, the bulk of pro-rPCSK4 stays in the ER/Golgi compartment where it may undergo partial processing or degradation. This is consistent with the predominantly perinuclear/cytoplasmic localisation of this protein when the cells were analysed by indirect immunofluorescence cytochemistry (Fig. 3B).

Figure 3A. *Intracellular compartmentalisation of rPCSK4 - Immunoblot analysis.* Stable HEK293 cells expressing rPCSK4FL-V5 were grown at either 37°C or 25°C to attenuate protein expression. Lysates were fractionated into nuclear/mitochondrial pellet (P15: pellet at 15 000 x g), ER/Golgi and microsomal pellet (P100: pellet at 100 000 x g), and soluble fraction (S100: supernatant at 100 000 x g) by differential centrifugation. These fractions were analysed by immunoblotting using the anti-V5 antibody.

3A

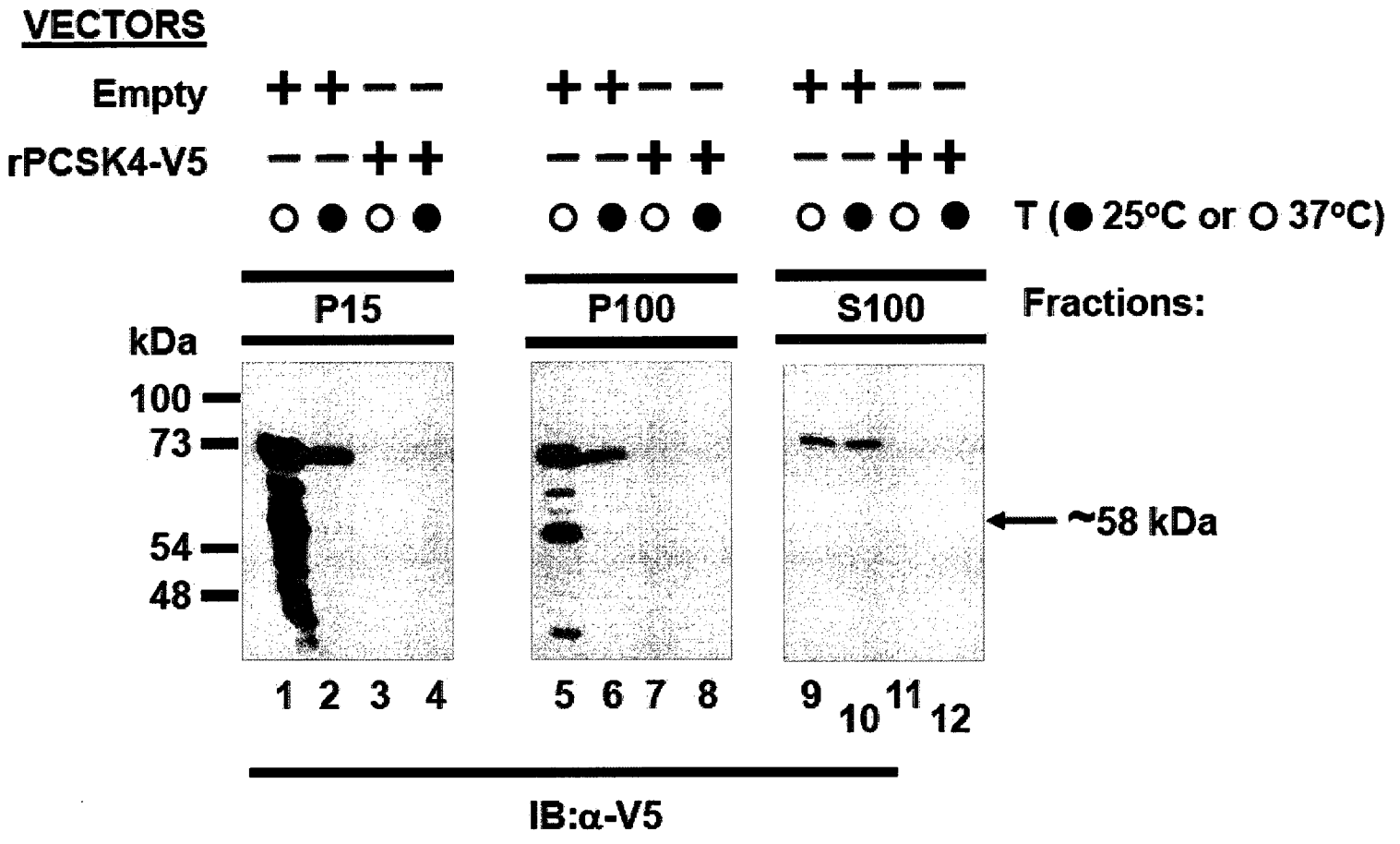
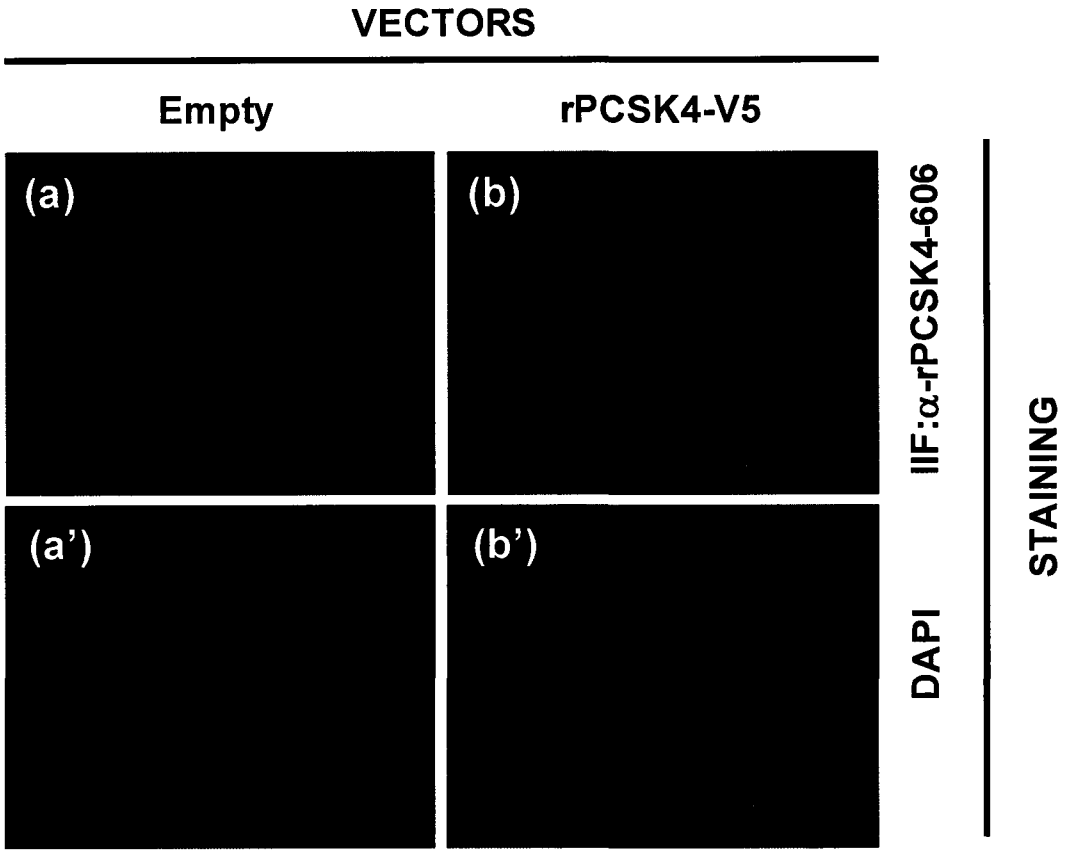

VECTORS
Empty
rPCSK4-V5
T (● 25°C or O 37°C)
P15
P100
S100
Fractions:
kDa
100
73
54
48
~58 kDa
1 2 3 4
5 6 7 8
9 10 11 12
IB:α-V5

Figure 3B. *Intracellular compartmentalisation of rPCSK4 - Indirect immunofluorescence analysis.* Indirect immunofluorescence was carried out by sequentially incubating paraformaldehyde permeabilised HEK293(rPCSK4FL-V5) cells with NHS, α-rPCSK4-606, and Alexa fluor 594 goat anti-rabbit IgG. After the application of drops of Vectashield Mounting Medium with DAPI, slides were examined under a Zeiss Axioplan epifluorescence microscope. Cells in (a) were transfected with the pCIneo empty vector and those in (b) with the pCIneo-rPCSK4FL-V5 vector. (a') is the corresponding DAPI staining of (a) and (b') is the corresponding DAPI staining of (b).

3B



3.3. Structure of human PCSK4

When studying rPCSK4 expression in HEK293 cells by ^{35}S-Met-Cys pulse-labelling followed by IP with α-rPCSK4NT, we consistently observed the presence of endogenous weakly immunoreactive bands of ~83 kDa and ~71-kDa, as well as a doublet of bands around ~66 kDa (Fig. 4A lane 1). Because the sizes of the two largest bands nearly corresponded to those predicted for human proPCSK4 and mature PCSK4, we suspected the expression of low amounts of this enzyme in these cells. RT-PCR on their total RNA using specific primers that should amplify a fragment of 678 bp confirmed the presence of *PCSK4* mRNA (Fig. 4B, lanes 1, 3 and 5). The full-length cDNA was amplified by long-range RT-PCR (Supplemental Fig. 1), cloned, and sequenced. The amino acid sequence derived from the cDNA ORF was identical to the one deposited in the database (ncbi accession #: Q6UW60), except for the presence of a Pro instead of a Ser at position 66. This substitution was judged benign by Polyphen analysis (http://coot.embl.de/PolyPhen/). Fig. 5 shows the alignment of hPCSK4 with its mouse and rat orthologues. Except for a single 2-aa gap between G^{481} and L^{482}, the sequences are continuously aligned and highly conserved from the start of prodomain (aa 28) to shortly past the Tyr-rich hydrophobic motif in the CT domain (aa 28-593). The downstream region is highly divergent. In human, it is 162 aa in length; 53 aa in rat; and 63 aa in mouse. It also contained a recognisable TM domain (aa 709-729) not seen in rodent orthologues.

Figure 4. *PCSK4 is endogenously expressed in HEK293 cell line.* **A)** HEK293 cells were transiently transfected with the two vectors: empty and rPCSK4FL-V5 and metabolically pulse-labelled with ^{35}S-Met-Cys for 4 h. Radiolabelled proteins in spent media and cell lysates were immunoprecipitated with α-rPCSK4NT antibody, resolved by 8.5% Tricine SDS-PAGE, and visualised by phosphorimaging. [Open and small arrows point to bands that represent possible endogenous expression of human PCSK4 in HEK293 cells. Wide arrow is the expressed rPCSK4-V5]. **B)** Three separate 50-µl PCRs were conducted. Each contained 1 µl of reverse-transcribed cDNA produced from total RNA extracted from HEK293 cells transfected with pCIneo empty vector or HEK293 cells transfected with rPCSK4-V5 or untransfected HEK293 cells; 5% dimethylsulfoxide (DMSO); 1 µM oligodeoxynucleotide (oligo) primers (sense: 5'-ttcatgtccacccacttctg-3'; antisense: 5'-tcgctttctgagctgacaa-3'); 2.5 units of *Taq* DNA polymerase (Invitrogen). The mixture was subjected to 40 amplification cycles each comprising of 30s at 94°C, 30s at 60°C, and 45s at 72°C. The expected 678-bp cDNA amplicons encoding hPCSK4$^{538–755}$ was obtained, confirming the presence of transcripts for this enzyme in HEK293 cells.

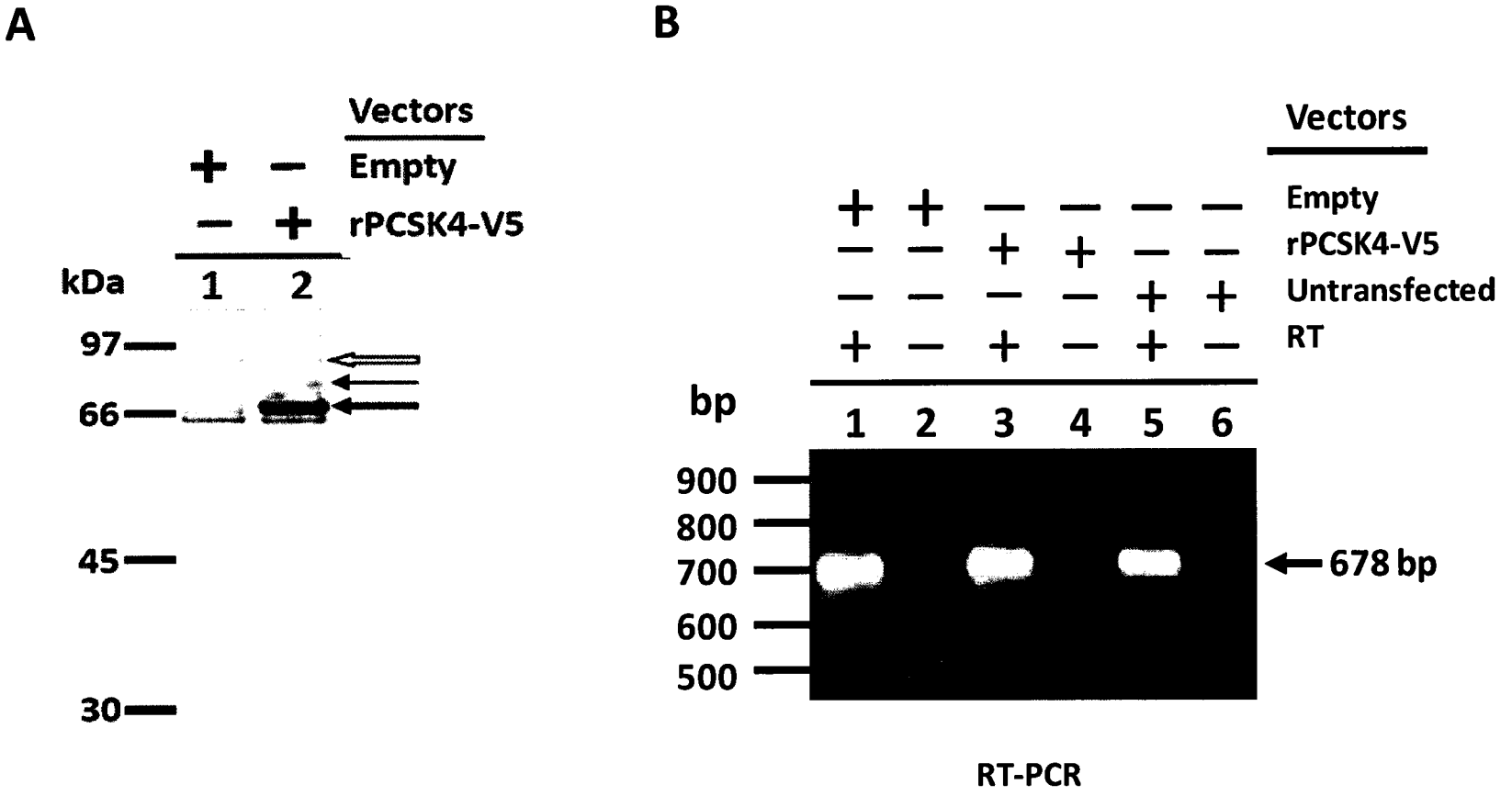
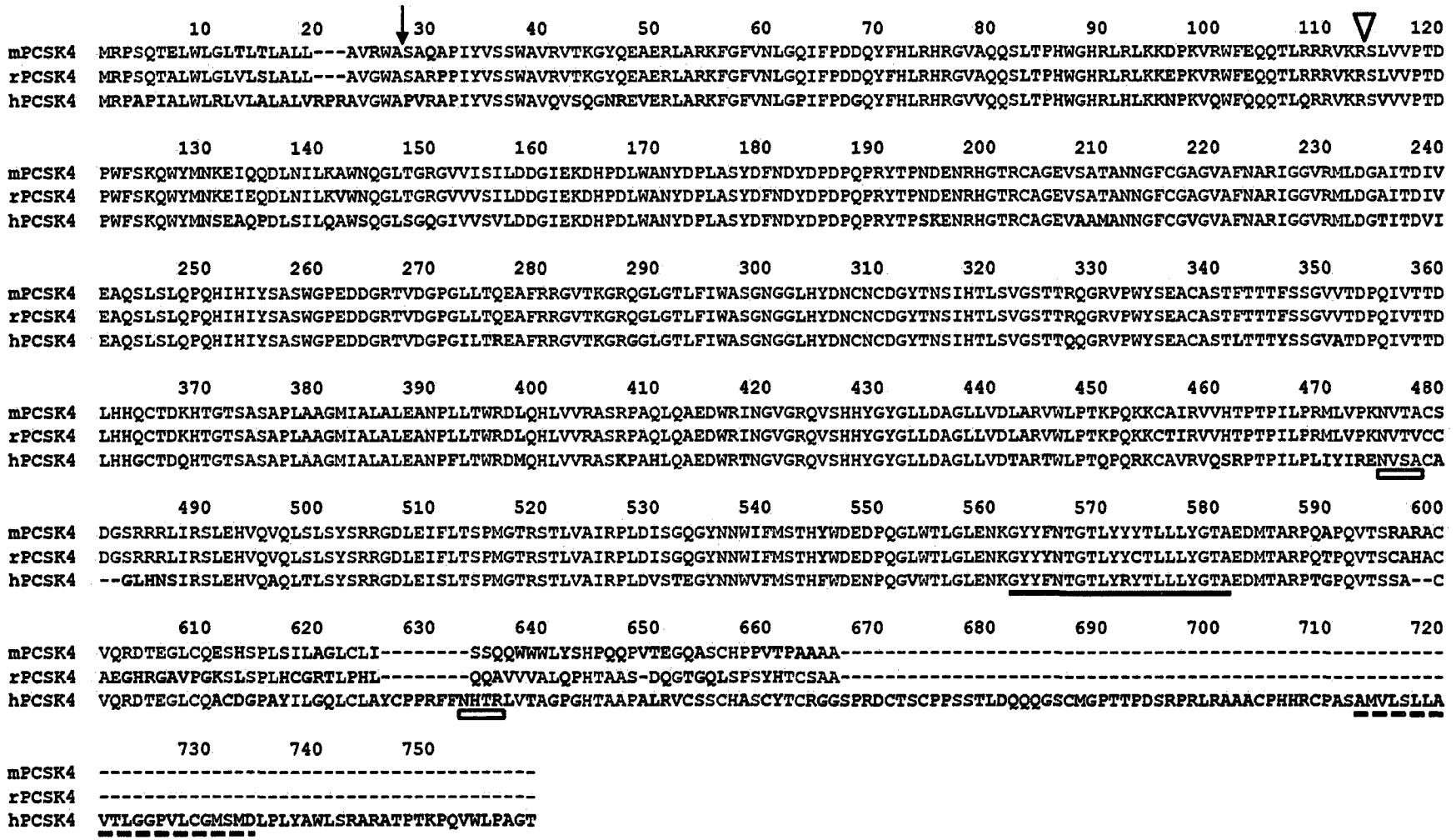

A
Vectors
+ − Empty
− + rPCSK4-V5
kDa
1 2
97
66
45
30
IP:α-rPCSK4NT
B
Vectors
+ + − − − − Empty
− − + + − − rPCSK4-V5
− − − − + + Untransfected
+ − + − + − RT
bp
1 2 3 4 5 6
900
800
700
600
500
678 bp
RT-PCR

Figure 5. *Alignment of hPCSK4 with its mouse and rat orthologues.* HEK293 cells hPCSK4 sequence was aligned with its mouse and rat orthologues: signal peptidase cleavage site is indicated by an arrow; primary prosegment cleavage site is shown by open arrow head; canonical motifs for N-glycosylation are underlined with open boxes; a 20-aa hydrophobic domain stretch is boldly underlined; a putative TM is underlined with broken line. Except for a single 2-aa gap between G^{481} and L^{482}, the sequences are continuously aligned and highly conserved, but the downstream region of the CT domain is highly divergent. In human, it is 162 aa in length, 53 aa in rat, and 63 aa in mouse. It also contained a recognisable TM domain (aa 709-729) not seen in rodent orthologues.


10 20 30 40 50 60 70 80 90 100 110 120
mPCSK4 MRPSQTELWLGLTLTLALL---AVRWASAQAPIYVSSWAVRVTKGYQEAERLARKFGFVNLGQIFPDDQYFHLRHRGVAQQSLTPHWGHRLRLKKDPKVRWFEQQTLRRRVKRSLVVPTD
rPCSK4 MRPSQTALWLGLVLSLALL---AVGWASARPPIYVSSWAVRVTKGYQEAERLARKFGFVNLGQIFPDDQYFHLRHRGVAQQSLTPHWGHRLRLKKEPKVRWFEQQTLRRRVKRSLVVPTD
hPCSK4 MRPAPIALWLRLVLALALVRPRAVGWAPVRAPIYVSSWAVQVSQGNREVERLARKFGFVNLGPIFPDGQYFHLRHRGVVQQSLTPHWGHRLHLKKNPKVQWFQQQTLQRRVKRSVVVPTD
130 140 150 160 170 180 190 200 210 220 230 240
mPCSK4 PWFSKQWYMNKEIQQDLNILKAWNQGLTGRGVVISILDDGIEKDHPDLWANYDPLASYDFNDYDPDPQPRYTPNDENRHGTRCAGEVSATANNGFCGAGVAFNARIGGVRMLDGAITDIV
rPCSK4 PWFSKQWYMNKEIEQDLNILKVWNQGLTGRGVVVSILDDGIEKDHPDLWANYDPLASYDFNDYDPDPQPRYTPNDENRHGTRCAGEVSATANNGFCGAGVAFNARIGGVRMLDGAITDIV
hPCSK4 PWFSKQWYMNSEAQPDLSILQAWSQGLSGQGIVVSVLDDGIEKDHPDLWANYDPLASYDFNDYDPDPQPRYTPSKENRHGTRCAGEVAAMANNGFCGVGVAFNARIGGVRMLDGTITDVI
250 260 270 280 290 300 310 320 330 340 350 360
mPCSK4 EAQSLSLQPQHIHIYSASWGPEDDGRTVDGPGLLTQEAFRRGVTKGRQGLGTLFIWASGNGGLHYDNCNCDGYTNSIHTLSVGSTTRQGRVPWYSEACASTFTTTFSSGVVTDPQIVTTD
rPCSK4 EAQSLSLQPQHIHIYSASWGPEDDGRTVDGPGLLTQEAFRRGVTKGRQGLGTLFIWASGNGGLHYDNCNCDGYTNSIHTLSVGSTTRQGRVPWYSEACASTFTTTFSSGVVTDPQIVTTD
hPCSK4 EAQSLSLQPQHIHIYSASWGPEDDGRTVDGPGILTREAFRRGVTKGRGGLGTLFIWASGNGGLHYDNCNCDGYTNSIHTLSVGSTTQQGRVPWYSEACASTLTTTYSSGVATDPQIVTTD
370 380 390 400 410 420 430 440 450 460 470 480
mPCSK4 LHHQCTDKHTGTSASAPLAAGMIALALEANPLLTWRDLQHLVVRASRPAQLQAEDWRINGVGRQVSHHYGYGLLDAGLLVDLARVWLPTKPQKKCAIRVVHTPTPILPRMLVPKNVTACS
rPCSK4 LHHQCTDKHTGTSASAPLAAGMIALALEANPLLTWRDLQHLVVRASRPAQLQAEDWRINGVGRQVSHHYGYGLLDAGLLVDLARVWLPTKPQKKCTIRVVHTPTPILPRMLVPKNVTVCC
hPCSK4 LHHGCTDQHTGTSASAPLAAGMIALALEANPFLTWRDMQHLVVRASKPAHLQAEDWRTNGVGRQVSHHYGYGLLDAGLLVDTARTWLPTQPQRKCAVRVQSRPTPILPLIYIRENVSACA
490 500 510 520 530 540 550 560 570 580 590 600
mPCSK4 DGSRRRLIRSLEHVQVQLSLSYSRRGDLEIFLTSPMGTRSTLVAIRPLDISGQGYNNWIFMSTHYWDEDPQGLWTLGLENKGYYFNTGTLYYYTLLLYGTAEDMTARPQAPQVTSRARAC
rPCSK4 DGSRRRLIRSLEHVQVQLSLSYSRRGDLEIFLTSPMGTRSTLVAIRPLDISGQGYNNWIFMSTHYWDEDPQGLWTLGLENKGYYYNTGTLYYCTLLLYGTAEDMTARPQTPQVTSCAHAC
hPCSK4 --GLHNSIRSLEHVQAQLTLSYSRRGDLEISLTSPMGTRSTLVAIRPLDVSTEGYNNWVFMSTHFWDENPQGVWTLGLENKGYYFNTGTLYRYTLLLYGTAEDMTARPTGPQVTSSA--C
610 620 630 640 650 660 670 680 690 700 710 720
mPCSK4 VQRDTEGLCQESHSPLSILAGLCLI--------SSQQWWWLYSHPQQPVTEGQASCHPPVTPAAAA----------
rPCSK4 AEGHRGAVPGKSLSPLHCGRTLPHL--------QQAVVVALQPHTAAS-DQGTGQLSPSYHTCSAA----------
hPCSK4 VQRDTEGLCQACDGPAYILGQLCLAYCPPRFFNHTRLVTAGPGHTAAPALRVCSSCHASCYTCRGGSPRDCTSCPPSSTLDQQQGSCMGPTTPDSRPRLRAAACPHHRCPASAMVLSLLA
730 740 750
mPCSK4 ----------
rPCSK4 ----------
hPCSK4 VTLGGPVLCGMSMDLPLYAWLSRARATPTKPQVWLPAGT

3.4. Biosynthesis of hPCSK4

To better study the biosynthesis, processing, and transport of pro-hPCSK4 in HEK293 cells, we generated vectors for the expression of potential end-products of pro-hPCSK4 processing based on current knowledge of hPCSK3 processing [22]: prepro^{hPCSK4}-V5 (pphPCSK4-V5), a secretory propeptide C-terminally tagged with a V5 epitope; pre-hPCSK4 (hPCSK4Δpro), a secretory mature enzyme lacking the prodomain; prepro-hPCSK4ΔTM-H6, terminating before the TM and prepro-hPCSK4ΔDoP-H6 terminating after the P domain. These hPCSK4 proteins are diagrammatically shown in Fig. 6A.

These expression vectors were transfected into HEK293 cells and cell lysates were analysed by IB using the α-mPCSK4 pAb or the α-V5 mAb. Immunoreactive proteins of the following approximate MWs were observed (Fig. 6B): ~15/12 kDa for pphPCSK4-v5 (lanes 1 and 3); ~74 kDa for hPCSK4Δpro (lanes 2 and 3) kDa; ~80 kDa for hPCSK4ΔTM-H6 (lane 4), ~63 kDa for hPCSK4ΔDoP-H6 (lane 5). None of the above products was observed when ~83-kDa full-length hPCSK4 was transduced (lane 6), indicating lack of significant processing of the proenzyme. Furthermore, none of them was detected in spent media (not shown).

Biosynthesis of hPCSK4 was also examined by pulse-chase analysis. The expression vector for prepro-hPCSK4 was transiently transfected into HEK293 cells; the cells were metabolically pulse labelled for 15 min using ^{35}S-Met-Cys, and chased for 15 to 240 min in the absence of radioactive amino acids. PCSK4-related proteins were immunoprecipitated using the α-rPCSK4NT pAb and analysed by SDS-PAGE and fluorography. As shown in Fig. 7, an intense protein band of ~83-kDa was observed after pulse labelling (Fig. 7, lane 1); its intensity slightly decreased during the chase (Fig. 7, lanes 2-7). This band was followed

Figure 6. *Expected and observed hPCSK4 forms.* **A)** Diagrammatic representation of additional expression vectors generated to further elucidate the nature of the 83/81 kDa immunoreactive bands: hPCSK4^{WT} (hPCSK4-FL), full-length enzyme containing all the domains; prepro^{hPCSK4}-V5 (pphPCSK4-V5), a secretory propeptide C-terminally tagged with a V5 epitope; pre-hPCSK4 (hPCSK4-Δpro), a secretory mature enzyme lacking the prodomain; prepro-hPCSK4ΔTM-H6 (hPCSK4-ΔTM), a C-terminally H6-tagged enzyme terminating before the TM; prepro-hPCSK4ΔDoP-H6 (hPCSK4-ΔDoP), a C-terminally H6-tagged enzyme terminating after the P domain. **B)** Expression vectors were transiently transfected into HEK293 cells; proteins in cell lysates were resolved by 8.5% Tricine SDS-PAGE and electroblotted onto PVDF membrane; immunoblotting was carried out using anti-mPCSK4 pAb, anti-V5 mAb, or anti-α-tubulin mAb. [The identities of the bands above 95 kDa are not known, and I speculate them to be non-specific binding of the antibody to some endogenous protein].

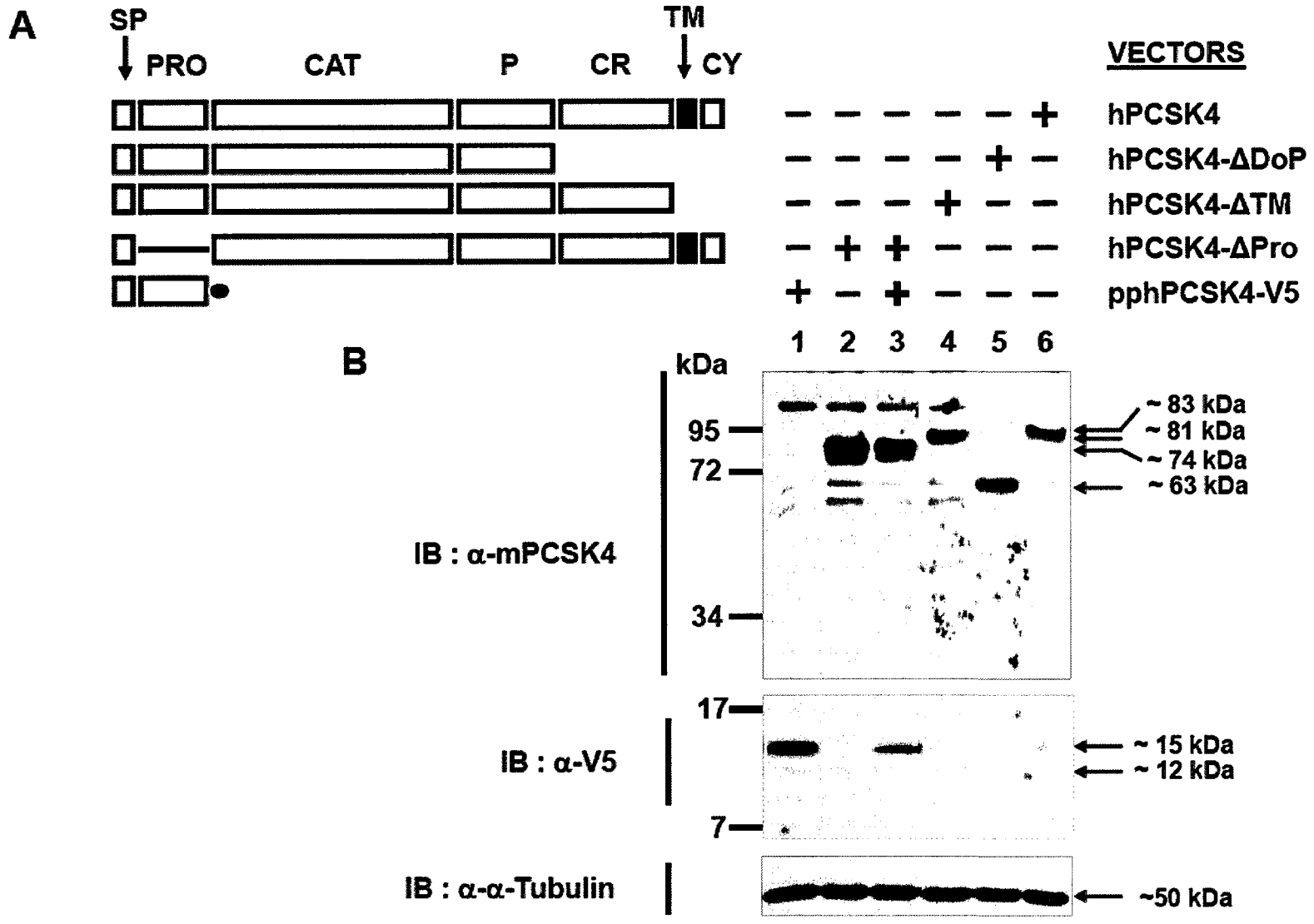
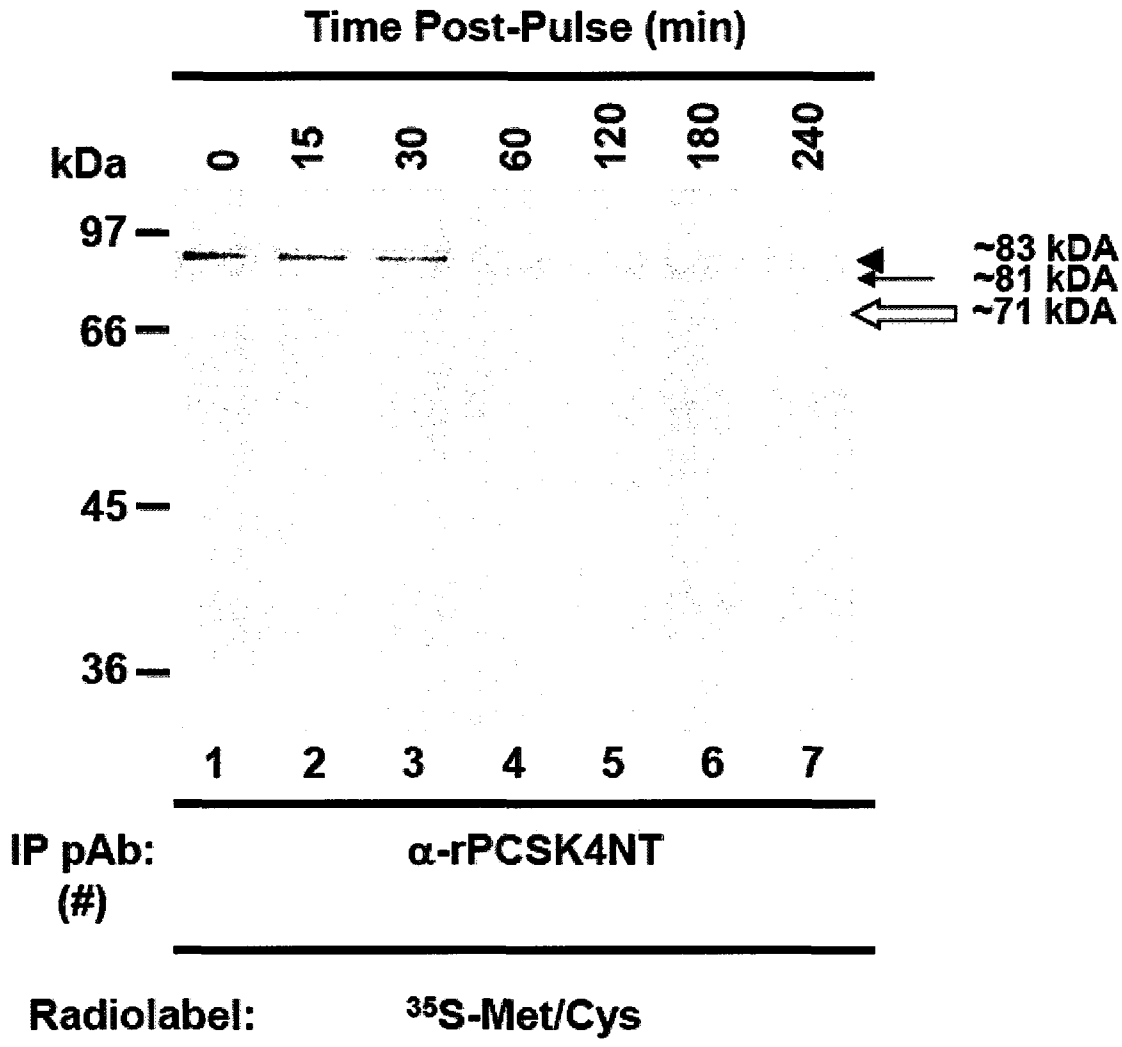

A
SP
PRO
CAT
P
CR
TM
CY
VECTORS
hPCSK4
hPCSK4-ΔDoP
hPCSK4-ΔTM
hPCSK4-ΔPro
pphPCSK4-V5
1 2 3 4 5 6
B
kDa
95
72
34
17
7
IB : α-mPCSK4
IB : α-V5
IB : α-α-Tubulin
~ 83 kDa
~ 81 kDa
~ 74 kDa
~ 63 kDa
~ 15 kDa
~ 12 kDa
~50 kDa

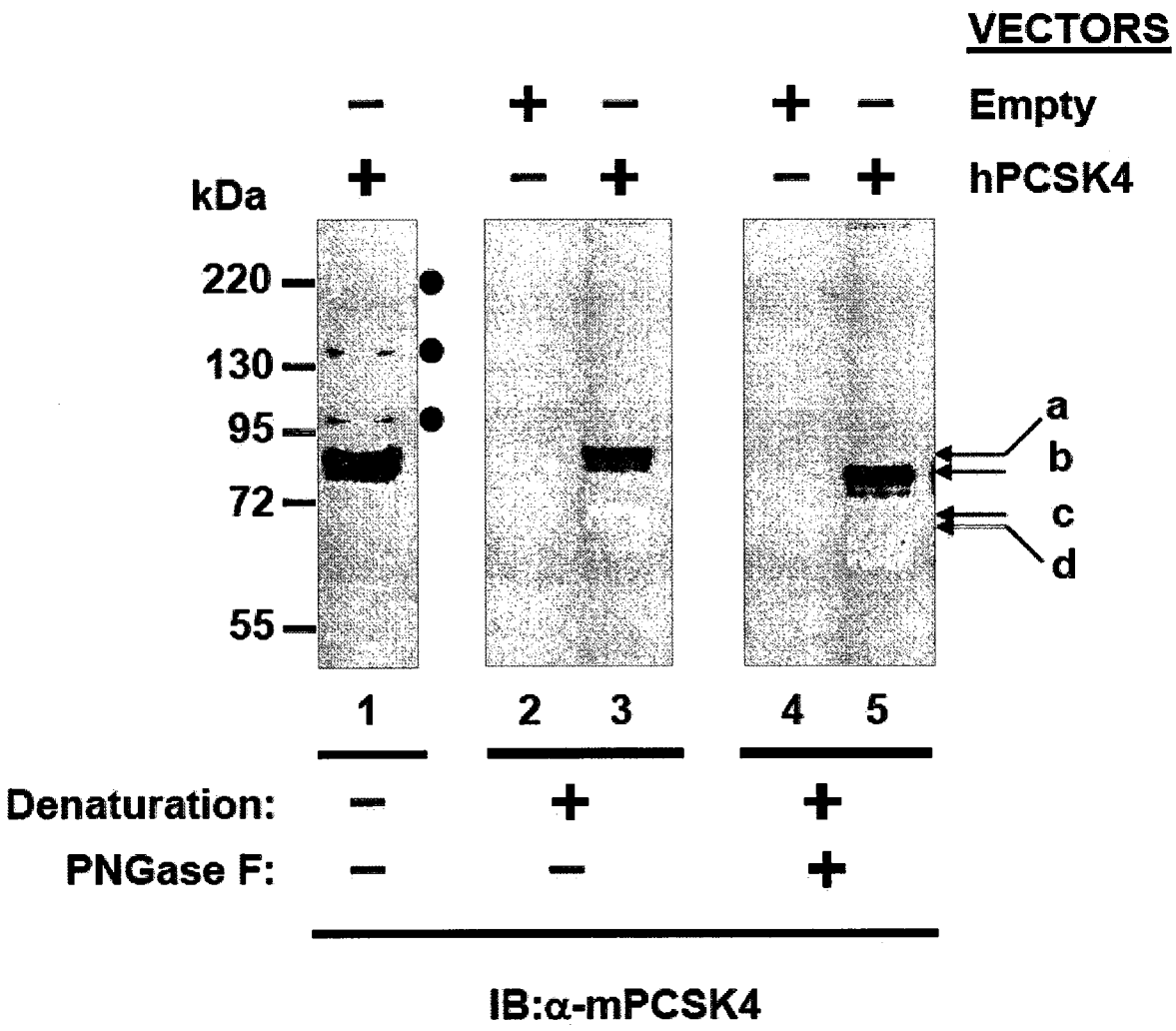
Figure 7. *Biosynthesis of hPCSK4.* Expression vector for prepro-hPCSK4 was transiently transfected into HEK293 cells, metabolically pulse-labelled for 15 min using ^{35}S-Met-Cys, and chased for varying times between 15 to 240 min in the absence of radioactive amino acids. hPCSK4-related proteins were immunoprecipitated using the α-rPCSK4NTpAb, resolved by 8.5% Tricine SDS-PAGE, and visualised by phosphorimaging. [The ~83 and 81-kDa bands were both identified to be PCSK4 and the ~71-kDa band as GRP78].


Time Post-Pulse (min)
kDa
0
15
30
60
120
180
240
97
66
45
36
~83 kDA
~81 kDA
~71 kDA
1
2
3
4
5
6
7
IP pAb:
(#)
α-rPCSK4NT
Radiolabel:
35S-Met/Cys

by a fainter band of about ~81 kDa which also slightly decreased during the chase. A third faint band of ~71 kDa was observed and its intensity increased during chase. The predicted molecular weights of pro-hPCSK4 and the prodomain-freed mature hPCSK4, both N-glycosylated at its two sites, are ~83 kDa and ~71 kDa, respectively; suggesting that the observed 71 kDa band may result from the processing of the larger bands. None of these proteins was detected in spent media (not shown).

To begin to unravel the identities of the hPCSK4-related bands, we first examined whether the 83/81 kDa doublet represented glycosylated and non-glycosylated forms of the same protein. We incubated lysates of cells transfected with either an empty vector or hPCSK4 in glycosylation buffer, supplemented or not with PNGase F for 24 h. They were then analysed by IB using the α-mPCSK4 pAb. Before incubation, the lysates from hPCSK4-expressing cells contained, in addition to the 83/81-kDa doublet (Fig. 8, lanes 3 and 5, bands a and b), immunoreactive molecular forms of ~110, ~150 and ~250 kDa (Fig. 8, lane 1, dots), which may represent hPCSK4 aggregates. Such aggregates have also been observed in cells transduced with rPCSK4 (see Supplemental Fig. 2). These forms disappeared upon incubation of the lysates in deglycosylation buffer, leading to the appearance of three bands of ~68, ~65 and ~60 kDa (Fig. 8, lanes 3 and 5, bands c, and d). Treatment with PNGase F reduced the size of bands ~83- and ~81-kDa to bands of ~75- and ~73-kDa, respectively (Fig. 8, compare lane 5 to lane 3). The 68 and 65-kDa proteins (c and d) disappeared following treatment with the glycosylation enzyme; this was associated with accumulation of proteins migrating at ~60 and ~58 kDa (Fig. 8, compare lane 5 to lane 3). The 8-kDa size reduction of hPCSK4 forms following deglycosylation suggests that these molecules are N-glycosylated at the canonical motifs N-X-S/T-X constituted by aa 475-480 (NVSA) and aa 629-632 (NHTR) (see Fig. 5).

Figure 8. *Identities of hPCSK4-related bands (83/81 kDa doublet).* Lysates of cells transfected with either empty vector or hPCSK4 were incubated in the absence or presence of PNGase F for 24 h. Proteins in lysates were resolved by SDS-PAGE and immunoblot analysis carried out using the α-mPCSK4 pAb. Dots represent immunoreactive molecular forms of possible hPCSK4 aggregates of ~110, ~150 and ~250 kDa which disappeared upon incubation of the lysates in deglycosylation buffer. The disappearance led to the appearance of three bands of ~68 ("c"), ~65 ("d") and ~60 kDa (not labelled). Treatment with PNGase F reduced the sizes of the ~83/81-kDa doublet ("a") to ~75/73-kDa doublet ("b").

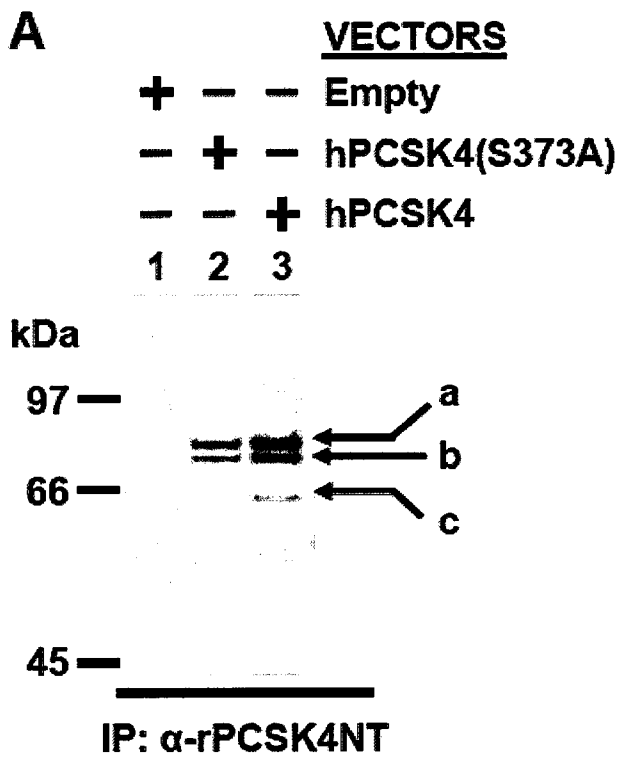

VECTORS
Empty
hPCSK4
kDa
220
130
95
72
55
a
b
c
d
1
2
3
4
5
Denaturation:
PNGase F:
IB:α-mPCSK4

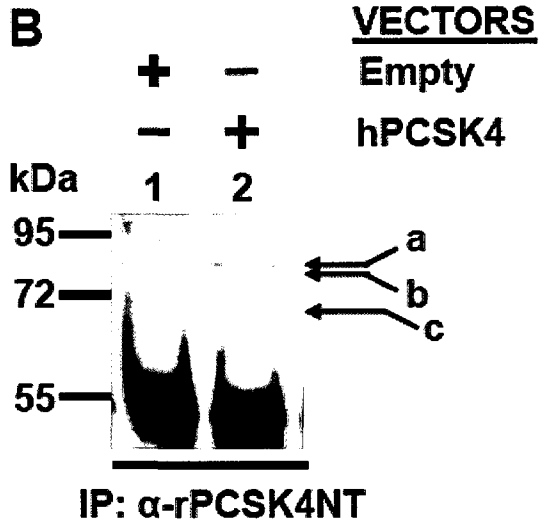
3.5. PCSK4 is associated with GRP78/BiP

During pro-PCSK maturation, the removal of the propeptide is an autocatalytic process that can be abrogated when one of the three residues of the catalytic triad is mutated. To determine whether such a mutation in pro-hPCSK4 could lead to zymogen accumulation and loss of related smaller protein, we generated a vector for the expression of hPCSK4($S^{373}A$) in which the active site Ser was mutated to an Ala. This vector and 2 others (empty and $hPCSK4^{WT}$) were transiently transfected into HEK293; after metabolic labelling with ^{35}S-Met-Cys, lysates were subjected to IP using the α-rPCSK4NT antibody and the precipitates analysed by SDS-PAGE and phosphorimaging. The results are shown in Fig. 9A. The 83/81 doublet (bands 'a' and 'b') was observed in cells expressing either the WT form (lane 3) or the $S^{373}A$ mutant form of hPCSK4 (lane 2).

In all PCSK4-specific IPs on lysates of HEK293 cells expressing transduced hPCSK4, a ~66-71 kDa protein was consistently pulled down by the pAbs. This is illustrated in Fig. 9A in radiolabelled lysates (band 'c'), in Fig. 9B in a silver-stained preparative gel (band 'c'), and in Fig. 7. This band, together with the upper two bands 'a' and 'b' were digested in gel with trypsin and subjected to LC-MS/MS. Table 1 lists for each band the number of matching peptides, their sequence, as well as the overall score. Bands 'a' and 'b' were both identified as human proPCSK4; band 'c' corresponded to human GRP78/BiP. To confirm the interaction between hPCSK4 and this chaperone, lysates from cells transfected with expression vectors for $hPCSK4^{WT}$ or $S^{373}A$ variants were subjected to IP with α-GRP78/BiP pAb antibody followed by IB with α-mPCSK4 pAb. The results in Fig. 9C indicate that the chaperone can specifically pull down the pro-hPCSK4 doublet in all cases.

Figure 9. *PCSK4 associates with GRP78/BiP.* **A)** An expression vector, hPCSK4($S^{373}A$), in which the active site Ser is mutated to Ala was generated. The expression vectors: empty, hPCSK4($S^{373}A$), and $hPCSK4^{WT}$ were transiently transfected into HEK293 cells; metabolically pulsed-labelled with ^{35}S-Met-Cys for 4 h; cell lysates were analysed by immunoprecipitation using α-rPCSK4NTpAb followed by SDS-PAGE and phosphorimaging. **B)** The empty vector and $hPCSK4^{WT}$ were transiently transfected into HEK293 cells; cell lysates were analysed by immunoprecipitation using α-rPCSK4NT pAb followed by SDS-PAGE and silver-staining; silver-stained bands 'a', 'b', and 'c' were excised out; digestion with trypsin was carried out and samples subjected to LC-MS/MS. **C)** Expression vectors: empty, $hPCSK4^{WT}$, or hPCSK4($S^{373}A$) were transiently transfected into HEK293 cells and cell lysates were subjected to immunoprecipitation using α-GRP78/BiP pAb. Immunoprecipitated proteins were then separated by SDS-PAGE, electroblotted onto PVDF membrane, and immunoblotted using the α-mPCSK4 pAb.


A
VECTORS
+ – – Empty
– + – hPCSK4(S373A)
– – + hPCSK4
1 2 3
kDa
97
66
45
a
b
c
IP: α-rPCSK4NT


B
VECTORS
+ – Empty
– + hPCSK4
kDa
1 2
95
72
55
a
b
c
IP: α-rPCSK4NT

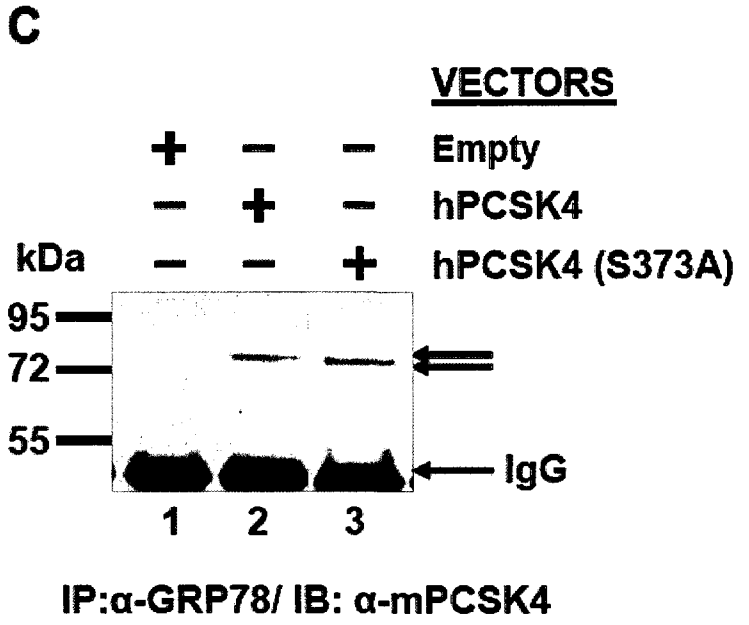

C
VECTORS
+ – – Empty
– + – hPCSK4
kDa – – + hPCSK4 (S373A)
95
72
55
IgG
1 2 3
IP:α-GRP78/ IB: α-mPCSK4

Table 1. *Proteins identified by LC-MS/MS.* LC-MS/MS was used to identify ~66-kDa protein consistently pulled down by various anti-PCSK4 antibodies; gi#, GenBank accession number.

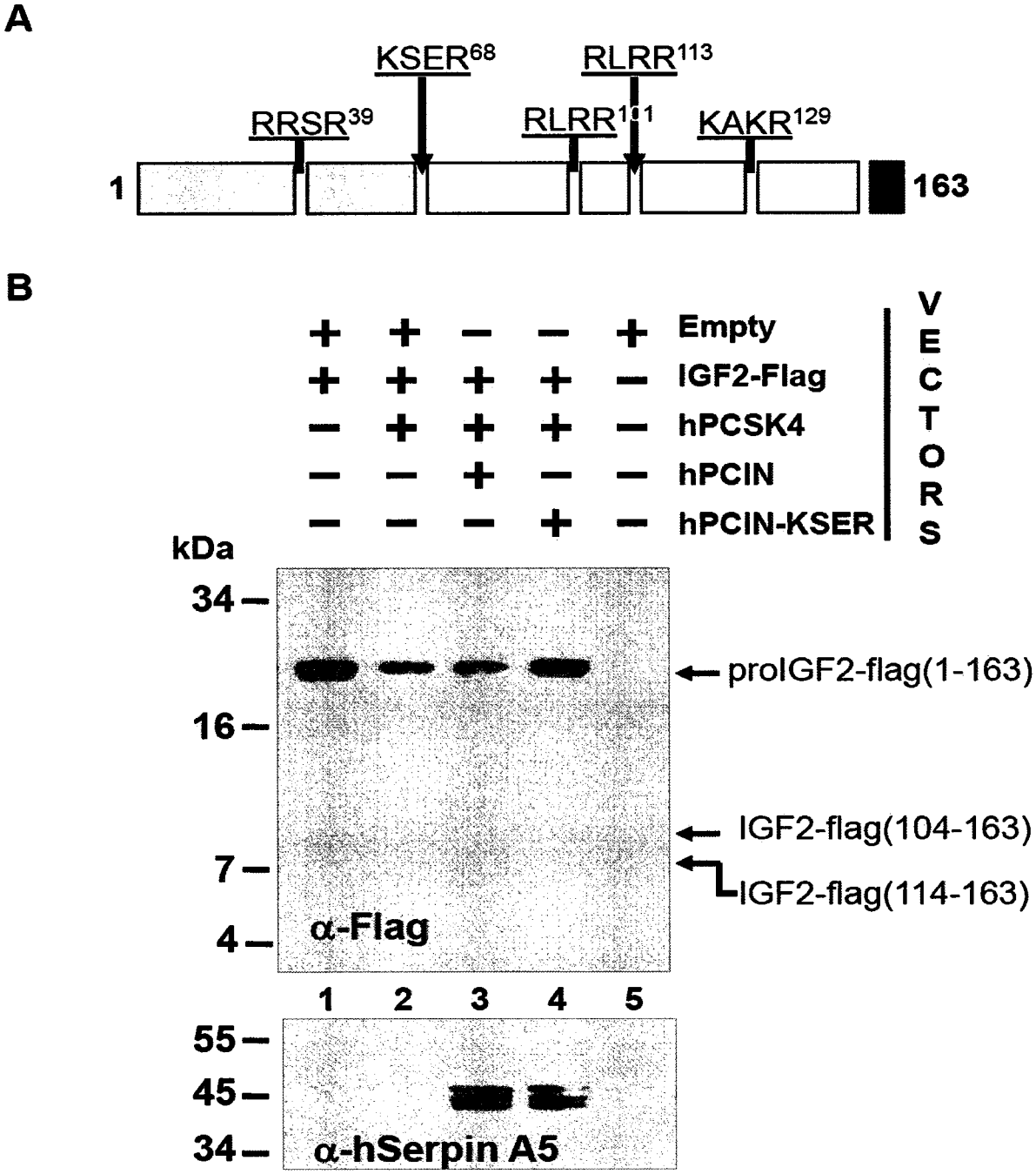
Band	gi#	Mass	Score	Peptides	Protein
a	76443679	84044	366	KVQWFQQQ	Proprotein convertase subtilin/kexin type 4 (Human)
				RTVDGPGI	
				RSLEHVQA	
				RATPTKPQ	
				RTWLPTQP	
				RLVTAGPG	
b	37183044	84044	167	KVQWFQQQ	Prohormone convertase subtilisin/kexin type 4 (Human)
				RSLEHVQA	
				KGYYFNTG	
c	16507237	72402	1024	RVEIIAND	Heat Shock 70 kDa Protein 5 (Glucose Regulated Protein, 78 kDa) (Human)
				KDAGTIAG	
				KSDIDEIV	
				KLYGSAGP	
				RLTPEEIE	
				KITITNDQ	

3.6. The hPCSK4 transduced in HEK293 cells is active

ProIGF2 carries a number of basic motifs recognisable by Kexin-like proprotein convertases (Fig. 10A). Some of these sites have been shown to be cleaved by PCSK1, 3, 4, or 7 [14, 23]; but in a human placental cell line, cleavage after the $KSER^{68}$ motif leading to the production of fully matured IGF2 appears to be mediated by PCSK4 exclusively [14]. Consistent with this observation, *in vitro* studies have shown that PCSK4 cleaves better than other Kexin-like convertases after a P1 Arg which is preceded by a P4 Lys [17, 24, 25]. Furthermore, we have found that insertion of the KSER motif into the reactive site loop (RSL) of the hPCIN serpin resulted in a variant ($hPCIN^{KSER}$) capable of inhibiting hPCSK4 enzymatic activity.

To ascertain that, in spite of the slow maturation, active hPCSK4 can be generated in transfected somatic cells, proIGF2 C-terminally tagged with a Flag epitope was transiently co-expressed with hPCSK4 in HEK293 cells in the presence of hPCIN or $hPCIN^{KSER}$. Thirty-six hours later, spent media were collected and analysed by IB for Flag-tagged proteins. The results are shown in Fig. 10B. Expression of proIGF2 alone resulted in the secretion of substantial amount of the precursor form together with smaller peptides of ~7 and ~8 kDa, presumably representing flagged O-glycosylated products of partial cleavage after $KAKR^{129}$ and $RLRR^{113}$, respectively (Fig. 10B, lane 1). Co-expression of hPCSK4 resulted in the reduced secretion of the precursor form and virtual disappearance of the smaller peptides (Fig. 10B, lane 2). These hPCSK4-induced changes were maintained in the presence of hPCIN (Fig. 10B, lane 3), but were reversed in the presence of $hPCIN^{KSER}$. Collectively, these data suggest that pro-hPCSK4 can be converted to active hPCSK4 in HEK293 cells, albeit at a very slow rate.

Figure 10. *hPCSK4 activity is inhibited by a bioengineered serpin variant.* **A)** Diagrammatic representation of the basic motifs in ProIGF2 sequence recognisable by Kexin-like proprotein convertases. Cleavage after the $KSER^{68}$ motif leading to the production of fully matured IGF2 is mediated by PCSK4 exclusively. **B)** proIGF2 C-terminally tagged with a Flag epitope was transiently co-expressed with hPCSK4 in HEK293 cells in the presence of hPCIN or $hPCIN^{KSER}$. Thirty-six hours spent media were collected, proteins in 30 µl aliquots were resolved by 8.5% Tricine SDS-PAGE, electroblotted onto PVDF membrane, and analysed by immunoblotting using α-Flag M2 mAb.


A
KSER68
RLRR113
RRSR39
RLRR111
KAKR129
1
163
B
+ + − − + Empty
+ + + + − IGF2-Flag
− + + + − hPCSK4
− − + − − hPCIN
− − − + − hPCIN-KSER
VECTORS
kDa
34
16
7
4
α-Flag
proIGF2-flag(1-163)
IGF2-flag(104-163)
IGF2-flag(114-163)
1 2 3 4 5
55
45
34
α-hSerpin A5
IB: Media

4. Discussion

This study was undertaken to elucidate the biosynthesis and trafficking of PCSK4. It started as an effort to explain (i) the small size (~54 kDa) of mouse sperm PCSK4 which suggested C-terminal truncation [12, 15], and (ii) its localisation at the cell surface in the absence of any recognisable TM domain in its C-terminal region [11]. Solubilisation of mouse sperm PCSK4 was attempted using different extraction procedures as described by Shetty *et al.* [18]. The enzyme could be rendered soluble only in Celis buffer due primarily to the high concentration of urea (9.6 M). Failure to release PCSK4 from particulate extracts in the presence of 1 M NaCl, 1% Triton X-100, or by temperature-induced partition between aqueous and detergent-rich phase in 1.7% Triton X-114 (Fig. 1) suggests the enzyme is not an integral membrane-bound protein; neither does it associate with membranes through ionic interactions, nor possess a transmembrane hydrophobic segment or a lipid anchor such as glycosyl-phosphatidyl-inositol (GPI). It is possible that PCSK4 is attached through strong hydrophobic bonds to insoluble sperm membrane components or forms precipitable aggregates once dissociated from membranes.

We surmised that the 20-residue hydrophobic segment within the CT domain might be partly responsible for this association or aggregation. This possibility was dismissed after we observed that a truncated version of rPCSK4 lacking the 20-residue hydrophobic domain, like the full-length WT form, could not be detected in the culture medium of stably transfected HEK293 cells. Furthermore, these experiments revealed that the bulk of the rPCSK4 transduced in these cells remained as a zymogen (Fig. 2) within the perinuclear/cytoplasmic region, presumably in the ER or the Golgi apparatus (Fig. 3B).

The presence in HEK293 cells of *PCSK4* transcripts amplifiable by RT-PCR (see Fig. 2) as well as of ~83-kDa and ~71-kDa protein recognised by an antibody against rat PCSK4 (Fig. 4) led us to believe that these human cells should allow efficient biosynthesis, maturation, and activation of isospecific PCSK4. The hPCSK4 cDNA cloned from these cells contained an ORF that should specify a potential type-1 integral membrane ectoenzyme (Fig. 5). However, transient expression of this cDNA into HEK293 cells and pulse-chase experiments indicated that the ~83-kDa pro-hPCSK4, like pro-rPCSK4 in stable transfectants, was not efficiently converted to its mature form by autoproteolytic cleavage after the prodomain (Fig. 7). Interestingly, labelled immunoprecipitates revealed additional proteins of ~81 and ~66-71 kDa pulled down by an antibody against the N-terminal region of rPCSK4. These proteins may represent either processed forms of hPCSK4 or interacting proteins. When vectors for the expected forms of hPCSK4 were generated and expressed into HEK293 cells, none was secreted in the medium, and except for the ~60-kDa ΔCT form, none migrated on immunoblots with MW of the immunoprecipitated forms. LC-MS/MS indicated that the ~81-kDa protein was an alternate form of pro-hPCSK4 and not its mature form, and the ~66-71 kDa protein to be GRP78/BiP.

The ~81-kDa protein may represent a product of a single cleavage within the prodomain. The conserved $RLAR^{51-54}$ sequence represents a putative motif for such a cleavage since it would remove 27 aa (~3.0 kDa) from the N-terminus of the prodomain. This cleavage, if it occurs, is not autocatalytic since it is also observed with the active-site mutant form of pro-hPCSK4 (Fig. 9A). It is also consistent with the size of the minor processing product of V5-tagged hPCSK4 prosegment when expressed as a distinct entity (see Fig. 6B).

Based mostly on the model developed for PCSK3 [26, 27], after biosynthesis in the ER, proPCSKs undergo a primary autocatalytic cleavage after the prodomain; the propeptide remains attached to the mature enzyme and then travels in complex with the mature enzyme into downstream secretory compartments until a secondary autocatalytic cleavage within the prodomain releases the resulting peptides [27-32]. Autocatalytic cleavage at the internal site without prior cleavage at the C terminal of the prodomain occurs in pro-PCSK8 [33]. The partial processing we propose for pro-hPCSK4 could be mediated in trans by co-resident Kexin-like proprotein convertases such as PCSK3, PCSK5, or PCSK7 (www.ncbi.nlm.nih.gov/geo/).

The interaction of both the ~83- and the ~81-kDa pro-hPCSK4 with GRP78/BiP (Fig. 9C), suggests that these zymogens are retained in the ER where the chaperone resides. In the case of PCSK7, this interaction was shown to slow down the maturation of the zymogen and its exit from the ER [34]. Furthermore, it apparently prevents the aggregation of this PCSK when overexpressed in Chinese hamster ovary CHO cells [35]. We presume that this chaperone plays similar effects on pro-hPCSK4 when it is overexpressed in HEK293 cells. The retention slows down activation of the transduced zymogen, but does not abrogate it. The presence of the enzymatic activity, capable of processing proIGF2 (Fig. 10), in these cells expressing PCSK4 supports this conclusion. It may also be that PCSK4 is an ER/Golgi resident protein in HEK293 cells.

One characteristic PCSK4 shares with PCSK7 is elevated expression in testicular germ cells [36, 37]. Calmegin, a calnexin-like protein, is an integral membrane-bound luminal chaperone found in abundance in the ER lumen of spermatocytes and spermatids [38]. Inactivation of its gene in mouse causes abnormal folding and trafficking of several secretory precursors necessary for the fertilising ability of sperm [38]. It is possible that, unlike

GRP78/BiP, calmegin may more efficiently mediate the folding of the proenzyme, allowing its faster maturation and exit from the ER. Co-expression of calmegin with pro-hPCSK4 however did not improve or facilitate the trafficking of pro-hPCSK4.

ACKNOWLEDGEMENTS

The authors thank Ms. Adrianna Gambarotta, Mr. Pierre Bradley, and Mr. Garnet Rodger for their help in animal maintenance as well as the immunisation of rabbits for antibodies production; Ms Haidy Tadros for her initial work in the design of pCIneo-rPCSK4-V5 and pCIneo-rPCSK4ΔCT-V5 vectors; Dr. Stephen J. Duguay (Genzyme Corp, Cambridge, MA) for providing us with the expression vector for proIGF2; Dr. Masaru Okabe (Genome Information Research Center, Osaka University, Japan) for the expression vector of calmegin.

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APPENDIX

1. Suplemental Materials and Methods

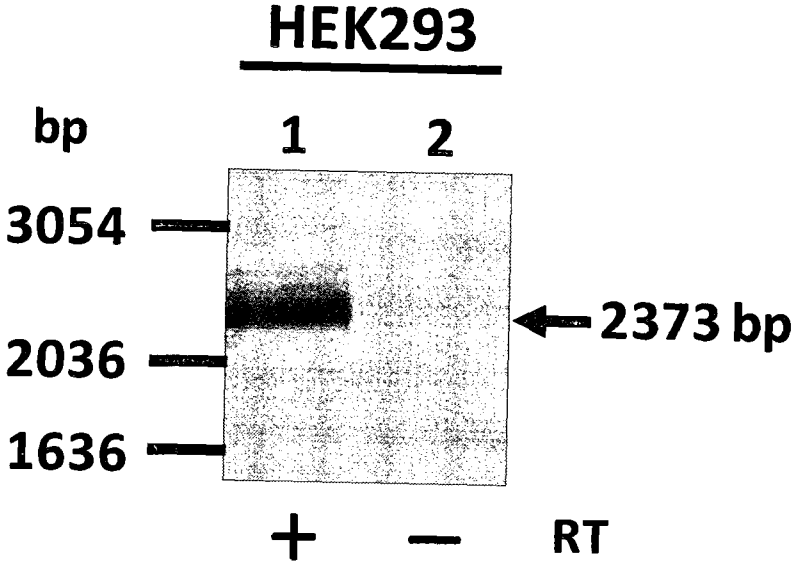
1.1. Amplification and cloning of hPCSK4 cDNA

Total RNA (RNA) was isolated from HEK293 cells using RNeasy Mini Kit as described by the manufacturer (Qiagen, Mississauga). To remove contaminating DNA, 10 μg of RNA was incubated at 37°C for 10 min in a 25-μl reaction mixture containing 6 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.5), and 0.5 μl of 10 U/μl DNAase I. The enzyme was inactivated with 2.5 μl of 50 mM EDTA (pH 7.5) by heating at 65°C for 10 min. After a quick chill on ice, 22 μl of the reaction mixture was supplemented with 2 μl of 0.5 μg/μl oligo(dT) and 2 μl of 10 mM dNTPs. After a 5-min heating for 5 min to undo secondary structures, 8 μl 5x First-Strand Buffer, 4 μl 0.1 M DTT, and 0.2 μl RNAse Inhibitor (Invitrogen) were added. The mixture was divided as 19-μl aliquots into two PCR tubes (R+, R-). The tubes were placed in a MasterCycler Gradient PCR machine (Eppendorf) and incubated at 42°C for 2 min; 1 μl SuperScript II reverse transcriptase (RT, Invitrogen) was added to the R+ content; both samples were allowed to incubate for 50 min at 42°C; the RT was inactivated by a 1-min incubation at 70°C.

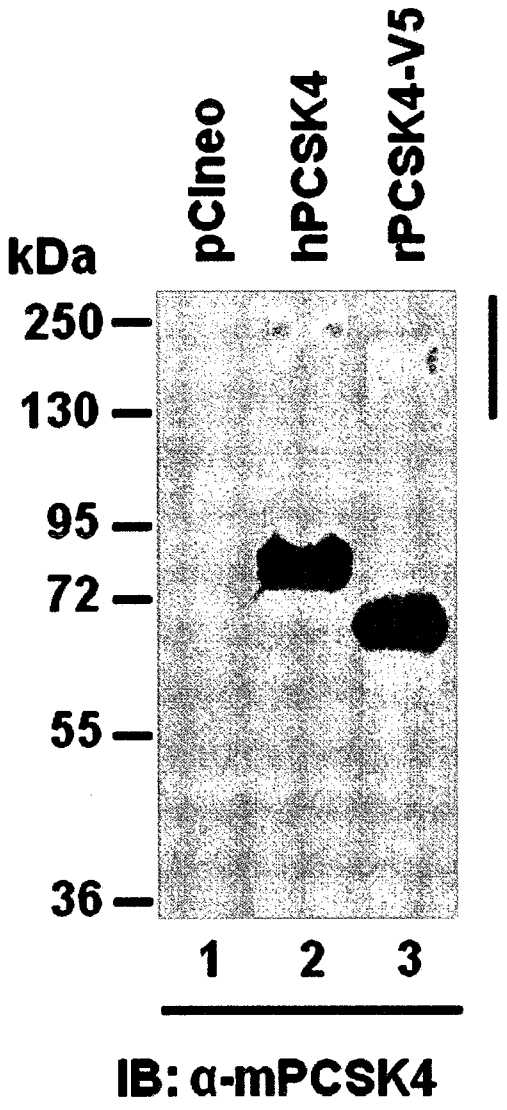
To amplify the complete ORF of hPCSK4 cDNA, a second PCR was performed for 35 cycles (each comprising incubation for 30s at 94°C, 30s at 55°C, 2.5 min at 68°C) in a 50 μl reaction buffer containing Elongase Enzyme Mix (Invitrogen), 5% bovine serum albumin, 5% DMSO, 5% glycerol, and 1 μM oligo primers (sense: 5'-acc tct aga gat gcg gcc cgc ccc gat t-3'; antisense: 5'- agc ggc cgc agg tcg ctt tct gag ctg ac-3', carrying *Xba*I and *Not*I restriction sites at their 5' end, respectively). The 2373-bp cDNA amplicon (Supplemental

Figure 1) was cleaned using PureLink PCR Purification Kit (Invitrogen). In parallel, this amplicon and the pCIneo mammalian expression vector (Promega) were successively digested with *Xba*I and *Not*I to generate the corresponding cohesive termini. The linearised vector was purified by agarose gel electrophoresis and extracted from the gel using the silica-based GeneClean Gel Isolation & Reaction Cleanup protocol (Q-Biogene Inc.). The digested cDNA amplicon was re-purified using the PureLink PCR Purification kit. Both were quantified by running aliquots on agarose gel beside DNA mass ladders (Invitrogen). Using 3 U of T4 DNA ligase (Roche Diagnostics, Mannheim, Germany), ligation was conducted in a 20-µl reaction mixture containing the amplicon and the linear plasmid in a 3:1 molar ratio. The mixture was incubated overnight at 14°C, diluted with an equal volume of water, and extracted with 10 volumes of 100% n-butanol to concentrate the DNA. The DNA pellet was rinsed with ice-cold 75% ethanol, air dried, and reconstituted in 10 µl H_2O. A 4-µl aliquot of the ligated DNA was electroporated into 40 µl of electro-competent XL1-Blue *E. coli* strain. The transformed bacteria were selected on LB/agar containing 100 µg/ml ampicillin. Plasmid DNAs, from selected positive clones were verified for size by agarose gel electrophoresis. Their cDNA inserts were sequenced in both directions on an automated sequencer using the various primers. The expression of the pCIneo-hPCSK4 plasmid alongside that of pCIneo-rPCSK4 is shown is Supplemental Fig. 2.

Supplemental Figure 1. *hPCSK4 is endogenously expressed in HEK293 cells.* Total RNA was isolated from 10^6 untransfected HEK293 (through RNeasy Mini Kit) and reversed transcribed into cDNA using reverse trancriptase (RT). By using the primers (sense: 5'-acc tct aga gat gcg gcc cgc ccc gat t-3'; antisense: 5'- agc ggc cgc agg tcg ctt tct gag ctg ac-3') and the reversed transcribed cDNA, a 2373 bp full-length hPCSK4 was amplified through PCR using Elongase (lane 1). Lane 2 represents the RT-negative control.


HEK293
bp
1
2
3054
2036
1636
2373 bp
+
—
RT

Supplemental Figure 2. *Does mature PCSK4 form higher M_r SDS-stable aggregates?* The expression vector pCIneo, pCIneo-hPCSK4, or pCIneorPCSK4-FL-V5 was transiently transfected into HEK293 cells. Aliquots of cell lysates were resolved by 8.5% Tricine SDS-PAGE, electroblotted onto PVDF membrane, and immunoblotted using α-mPCSK4 pAb.


pCIneo
hPCSK4
rPCSK4-V5
kDa
250
130
95
72
55
36
1
2
3
IB: α-mPCSK4

1.2. Plasmid vectors for expression of mutated forms hPCSK4

1.2.1. hPCSK4^{S373A}

The QuickChange Site Directed Mutagenesis Kit from Stratagene (La Jolla, CA) and the oligo pair (sense: 5'-cac acg ggc acc gcg gcc tca gcc c-3'; antisense: 5'-ggg ctg agg ccg cgg tgc ccg tgt g-3') were used following the manufacturer's protocol to mutagenise the cDNA for hPCSK4 to one specifying the active site mutant hPCSK4^{S373A}.

1.2.2 hPCSK4ΔTM-H6 and hPCSK4ΔDoP-H6

Vectors for expression of the deletion mutants, hPCSK4ΔDoP-H6 (aa 1 – 551) and hPCSK4ΔTM-H6 (aa 1 – 705), were constructed by digesting pCIneo-hPCSK4 with *Sal*I/*Not*I and *Age*I/*Not*I, respectively. The digested vectors were cleaned and quantified as already described for hPCSK4. They were separately ligated with the following pre-annealled sense and antisense oligos:

(a) for hPCSK4ΔDoP-H6:

sense: 5'-tcgaccggaggtggttcgcattcatcatcatcatgctgcttgaagttgtcagctcagaaagcgacctgc-3'

antisense: 5-ggccgcaggtcgctttctgagctgacaacttcaagcagcatgatgatgatgatgcgaaccacctccgg-3'

(b) for hPCSK4ΔTM-H6:

sense: 5'-ccggtgggttcgcatcatcatcatcatgctgcttgaagttgtcagctcagaaagcgacctgc-3'

antisense: 5'-ggccgcaggtcgctttctgagctgacaacttcaagcagcatgatgatgatgatgcgaaccaccgca-3'.

The double-stranded oligos, in addition to containing cohesive termini compatible with those of their respective linearised vectors also encoded the C-terminal hexahistidinyl (H6) motif-containing peptides STGGGSHHHHHHAA and GGSHHHHHAA, respectively for hPCSK4ΔDoP-H6 and hPCSK4ΔTM-H6. The subsequent steps to the final product were as described for hPCSK4.

1.2.3. pphPCSK4-V5

This cDNA fragment encoding the prepro domain of hPCSK4 was cloned into the backbone of the pIR-hPCSK9-V5 expression vector. This vector carries the cDNA for human PCSK9 with an in-frame 3'-DNA sequence encoding the simian virus 5 (V5) epitope in the multiple cloning site 1 (MCS1) of the bi-cistronic vector, pIRES2-EGFP (Clontech), and the cDNA for enhanced green fluorescent protein (EGFP) in its MCS2 [1, 2]. The cDNA for pphPCSK4-V5 was amplified through a 50-µl PCR reaction mixture containing 100 ng pCIneo-hPCSK4 as DNA template, 1 µM each of oligo primers (forward: 5'-gctagcgctatgcggcccgccccgatt-3'; reverse: 5'-agtaccggtcacgacagagcgtttcacc-3' containing *Nhe*I and *Age*I restriction sites, respectively), 5% DMSO, and 2.5 units of *Taq* DNA polymerase through 20 initial cycles each consisting of a 30s denaturation at 94°C, a 30s annealing at (70 ± 1)°C, and a 30s polymerisation at 72°C. This was followed by 30 additional cycles of 94°C for 30s, 51°C for 30s, and 72°C for 30s. The amplicon was digested with *Nhe*I and *Age*I and inserted into pIR backbone from which the hPCSK9 sequence had been excised out using the same enzymes, thus generating the pIR-pphPCSK4-V5 vector. The accuracy of the insert was confirmed by sequencing.

1.2.4. PCSK4Δpro

The pCIneo-hPCSK4 vector was digested with *Apa*I/*Pfl*MI removing the fragment encoding aa 27–125. The linear plasmid was purified and ligated with a double-stranded oligo with corresponding cohesive ends (sense: 5'-ccgtctgtcgtggtgcccacggacccctggttctc caagca-3' and antisense 5'-ttggagaaccaggggtccgtgggcaccacgacagacggggcc-3'); encoding the signal peptide directly joined to the catalytic domain to generate pCIneo-hPCSK4ΔPro. It was used to transform bacteria, subsequently extracted, purified, and accuracy of the insert verified by sequencing.

1.3. Plasmid vectors for expression of rPCSK4 fragments

1.3.1. pIVEX 2.4d vector

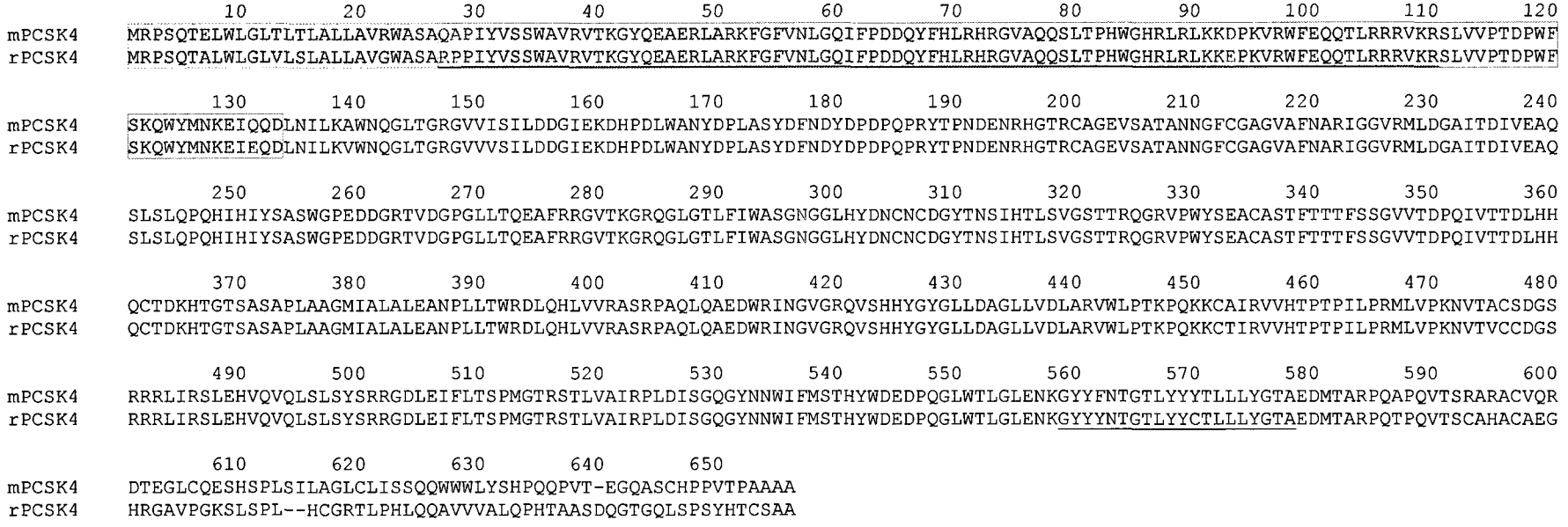
The sequences of oligo primers for PCR amplification of rPCSK4 cDNA fragments encoding an N-terminal (NT: aa 27-133) and C-terminal (CT: aa 444-580) regions of the enzyme are shown in Table 1. They were designed with a 5' tail containing a restriction site for cohesive end-generating enzyme to facilitate cloning, first, into the bacteria expression vector pIVEX 2.4d (Roche) which carries a sequence encoding a hexahistidinyl (H6) epitope upstream of the MCS. PCR was conducted in a 50-µl reaction mixture, containing 100 ng of pCIneo-rPCSK4-V5 as template, 1 µM each of forward and reverse primers, and 2.5 units of *Taq* DNA polymerase in 25 cycles including 30s at 94°C, 30s at 50°C or 52° (Supplemental Table 1 and Supplemental Figure 3), and 30s at 72°C. The cDNA amplicons were digested

with *Not*I and *Bam*HI and ligated into corresponding sites in an ORF with the H6-encoding segment, generating the pIVEX-H6rPCSK4NT and pIVEX-H6rPCSK4CT vectors. These vectors served as templates in a cell-free transcription/transcription system for the production of H6-tagged rPCSK4 fragments using the RTS 500 reaction device and ProteoMaster *E. coli* HY Kit from Roche.

Supplemental Table 1. *PCR Primers for rPCSK4 cDNA Fragment Amplification.* The forward (F) and reverse (R) primers were designed to amplify the selected rPCSK4 peptides from the corresponding cDNA. The region homologous to the cDNA is given in capital letters; a tetrapeptide encoded by the sequence is shown beneath it. A 5' tail containing a restriction enzyme cleavage site (single underline) is given in small letters; the cleaving enzyme is shown below. The stop codon in R primers is doubly underlined. The annealing temperature (Ta) for each primer pair is shown in column 1.

PCSK4 Region	*Primer Names*	*Sequence 5'→3'*
NT Pair (T_a:52°C)	**rPCSK4-RPPI-F/*Not*I**	**ccgcggccgcCGACCACCCATCTATGT** ***Not*I R P P I**
	rPCSK4-IEQD-R/*Bam*HI	**ccggatcctcAATCTTGTTCTATCTCCTT** ***Bam*HI D Q E I**
CT Pair (T_a:50°C)	**rPCSK4-LPTK-F/*Not*I**	**ccgcggccgcCTCCCTACTAGCCTCA** ***Not*I L P T K**
	rPCSK4-TAED-R/*Bam*HI	**ccggatcctcaGTCCTCTGCCGTCCCATA** ***Bam*HI D E A T**

Supplemental Figure 3. *Sequences of rPCSK4NT and rPCSK4CT fragments.* mPCSK4 sequence was aligned with the rat orthologues. His, Asn, Asp, and Ser residues forming the catalytic pocket are shown in red. Amino acids sequences selected for NT and CT fragments are highlighted in cyan and yellow, respectively.


10 20 30 40 50 60 70 80 90 100 110 120
mPCSK4 MRPSQTELWLGLTLTLALLAVRWASAQAPIYVSSWAVRVTKGYQEAERLARKFGFVNLGQIFPDDQYFHLRHRGVAQQSLTPHWGHRLRLKKDPKVRWFEQQTLRRRVKRSLVVPTDPWF
rPCSK4 MRPSQTALWLGLVLSLALLAVGWASARPPIYVSSWAVRVTKGYQEAERLARKFGFVNLGQIFPDDQYFHLRHRGVAQQSLTPHWGHRLRLKKEPKVRWFEQQTLRRRVKRSLVVPTDPWF
130 140 150 160 170 180 190 200 210 220 230 240
mPCSK4 SKQWYMNKEIQQDLNILKAWNQGLTGRGVVISILDDGIEKDHPDLWANYDPLASYDFNDYDPDPQPRYTPNDENRHGTRCAGEVSATANNGFCGAGVAFNARIGGVRMLDGAITDIVEAQ
rPCSK4 SKQWYMNKEIEQDLNILKVWNQGLTGRGVVVSILDDGIEKDHPDLWANYDPLASYDFNDYDPDPQPRYTPNDENRHGTRCAGEVSATANNGFCGAGVAFNARIGGVRMLDGAITDIVEAQ
250 260 270 280 290 300 310 320 330 340 350 360
mPCSK4 SLSLQPQHIHIYSASWGPEDDGRTVDGPGLLTQEAFRRGVTKGRQGLGTLFIWASGNGGLHYDNCNCDGYTNSIHTLSVGSTTRQGRVPWYSEACASTFTTTFSSGVVTDPQIVTTDLHH
rPCSK4 SLSLQPQHIHIYSASWGPEDDGRTVDGPGLLTQEAFRRGVTKGRQGLGTLFIWASGNGGLHYDNCNCDGYTNSIHTLSVGSTTRQGRVPWYSEACASTFTTTFSSGVVTDPQIVTTDLHH
370 380 390 400 410 420 430 440 450 460 470 480
mPCSK4 QCTDKHTGTSASAPLAAGMIALALEANPLLTWRDLQHLVVRASRPAQLQAEDWRINGVGRQVSHHYGYGLLDAGLLVDLARVWLPTKPQKKCAIRVVHTPTPILPRMLVPKNVTACSDGS
rPCSK4 QCTDKHTGTSASAPLAAGMIALALEANPLLTWRDLQHLVVRASRPAQLQAEDWRINGVGRQVSHHYGYGLLDAGLLVDLARVWLPTKPQKKCTIRVVHTPTPILPRMLVPKNVTVCCDGS
490 500 510 520 530 540 550 560 570 580 590 600
mPCSK4 RRRLIRSLEHVQVQLSLSYSRRGDLEIFLTSPMGTRSTLVAIRPLDISGQGYNNWIFMSTHYWDEDPQGLWTLGLENKGYYFNTGTLYYYTLLLYGTAEDMTARPQAPQVTSRARACVQR
rPCSK4 RRRLIRSLEHVQVQLSLSYSRRGDLEIFLTSPMGTRSTLVAIRPLDISGQGYNNWIFMSTHYWDEDPQGLWTLGLENKGYYYNTGTLYYCTLLLYGTAEDMTARPQTPQVTSCAHACAEG
610 620 630 640 650
mPCSK4 DTEGLCQESHSPLSILAGLCLISSQQWWWLYSHPQQPVT-EGQASCHPPVTPAAAA
rPCSK4 HRGAVPGKSLSPL--HCGRTLPHLQQAVVVALQPHTAASDQGTGQLSPSYHTCSAA

1.3.2. pET24b(+) vector

The yield in the pIVEX system was unsatisfactory, so the cDNA ORFs were excised out by digestion with *Xba*I and *Bam*HI and transferred into the same sites in the pET24b(+) inducible bacterial expression vector (Novagen, Gibbstown, NJ) to generate pET-H6rPCSK4NT and pET-H6rPCSK4CT vectors.

1.3.3. pCIneo vector

NT and CT rPCSK4 cDNA fragments were PCR amplified using pCIneo-rPCSK4-V5 as template and oligo primer pairs containing 5'*Nhe*I and *Eco*RI restriction sites in the sense and antisense sequences, respectively. The sense/antisense sequences were: for the NT amplicon, 5'-ctagctagcatggggccctcccagac-3'/5'-ccggaattctcaatcttgttctatctcc-3'; for the CT amplicon, 5'-ctagctagcatgctccctactaagcctca-3'/5'-ccggaattctcagtcctctgccgtcccata-3'. These amplicons were successively digested with *Nhe*I and *Eco*RI and subcloned into the same sites in pCIneo expression vector, generating the pCIneo-rPCSK4NT and pCIneo-rPCSK4CT vectors. These vectors were used in genetic vaccination of rabbits for antibody production.

1.4. Production and purification of recombinant H6-rPCSK4NT and H6-rPCSK4CT

pET-H6rPCSK4NT and pET-H6rPCSK4CT plasmids were used to transform thermo-competent Rosetta(DE3)pLysS *E. coli* cells (Novagen) as described by the manufacturer. Aliquots of positive clones were grown at 37°C with shaking at 230 rpm until

the optical density (OD) reached 0.6. 1mM Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) was added to induce expression of the recombinant protein and cells were allowed to grow for additional 4 h. They were then harvested by centrifugation and lysed in a buffer containing 6 M Guanidine-HCl; 0.1 M NaH_2PO_4; 0.01 M Tris-Cl, 300 mM NaCl; 20 mM Imidazole; 20 mM β-ME; 2 % TX-100; 9 % glycerol; 9 % ethanol; pH 8.0. The peptides were purified by affinity chromatography on a Ni-resin (Qiagen) as described by the manufacturer.

1.5. Expression vectors for human Protein C Inhibitor (hPCIN) and its hPCIN^{KSER} variant

Total human testicular mRNA was purchased from BD Biosciences Clontech (Palo Alto, CA) and reversed transcribed into cDNA as described above for hPCSK4. The hPCIN cDNA was amplified for 40 cycles (94°C for 10s, 52°C for 30s and 72°C for 10s) in a 50-µl PCR reaction mixture containing: 2.5 units of *Taq* DNA polymerase; 2 µl of total testicular cDNA, 1 µM of each oligo primer (sense, 5'-taggctagcatgcagctcttcctcctctt-3'; antisense, 5'-gaagcggccgctcaggggcggttcacttt-3' containing 5' sites for *Nhe*I and *Not*I sites, respectively. The cDNA amplicon was digested with these two enzymes and subcloned into the same sites of the pCIneo expression vector. The accuracy was confirmed by sequencing.

Two site-directed mutageneses were performed in the region specifying the reactive site loop (RSL) using the Stratagene kit: the first one conducted with the sense and antisense oligos 5'-cag gtc ggc ccg cct gaa Ttc tca gag gct agt gtt c-3' and 5'-gaa cac tag cct ctg aga A tt cag gcg ggc cga cct g-3' converted a gaaCtcc to the gaaTtcc *Eco*R1 site; the second one conducted with the sense and antisense oligos 5'-gggaaccagagcCgcggcagccacg-3' and 5'-

cgtggctgccgcGgctctg gttccc 3' converted a cAgcgg to cCgcgg *Sac*II site. The double-mutant was successively digested with *Eco*RI and *Sac*II to remove a fragment of RSL-encoding scissile sequence and substituted with a double-stranded oligo consisting of the following sense and antisense oligos 5'-ggcagccacgacccccgccaagtccgagagggacgtgcgcctg-3' and 5' aattcaggcgcacgtccctctcgga cttggcgggggtcgtggctgccgc-3'. The resulting pCIneohPCIN^{KSER} carried a cDNA sequence specifying a mutant PCIN with TPAKSE<u>R</u>DV$^{283-291}$ in its RSL (P1 of scissile bond underlined) instead of GTIFTF<u>R</u>SA$^{283-291}$ of wildtype hPCIN.

1.6. Antibody production

1.6.1 By vaccination with plasmid expression vector

Six-wk old female New Zealand White Rabbits (Charles River Laboratories, Montreal, QC) were tranquillised by subcutaneous (sc) injection of 0.2 – 0.3 ml 10 mg/ml Atravet for 15 min after which 5 ml blood was taken from each rabbit prior to the immunisation. The blood was used in the preparation of preimmune serum. Each rabbit was then immunised by sc injection with a total of 320 μg pCIneo-rPCSK4NT DNA in 800 μl of 0.15 N NaCl (normal saline) at 4 separate sites on the pre-shaved back. Boosting with the same quantity of vectors was carried out on wk 2, 4, and 19. They were bled by cardiac puncture on wk 21 and sera prepared.

1.6.2. By vaccination with recombinant peptides

Using a Kendall SolutionPlus® 3-Way Stopcock, 500 µl Complete Freund's Adjuvant were mixed with the 500 µl containing 200 µg H6rPCSK4NT peptide in normal saline until an emulsion was formed. The whole 1 ml emulsion was sc injected at 4 sites on the pre-shaved back of each rabbit. Boost injections were administered as above on wk 4, 8, 12, 19, 23, 27 and 31. Rabbits were bled by cardiac puncture and sera prepared.

The tested properties of the antibodies are summarised (Supplemental Table 2).

1.7. Silver staining protocol

Stock solutions were prepared as enumerated below:

i.	Silver stain fixing solution	[30% (v/v) ethanol, 5% (v/v) acetic acid, 1L]
ii.	Sensitising solution	[0.2% (w/v) sodium thiosulphate, 1L]
iii.	Silver solution	[0.2% (w/v) silver nitrate, 1L]
iv.	Developing solution	[4% (w/v) potassium carbonate, 0.025% (v/v)
v.	Stop solution	[4% (w/v) Tris, 2% Acetic acid 1L]

(Note: Within 1 h of use, add 0.025 % formaldehyde to developing solution)

Supplemental Table 2. *Properties of α-PCSK4 antibodies used in this study.* Summary of antibodies used in the study and the techniques in which they have been tested are depicted. Species tested: h, human; m, mouse. PCSK4-specificity: Y, yes; N, no

Name		Immunogen		Tested species		Tested Techniques				
						IB		IP		IHC
α-mPCSK4		$mPCSK4^{133-506}$		h, m		Y		Y		N
α-rPCSK4-606		$rPCSK4^{1-654}$		h, m		N		Y		Y
α-rPCSK4NT		$rPCSK4^{27-133}$		h, m		Y		Y		Y

The 1.5 mm SDS-PAGE was silver stained following the sequence below:

1.	Silver staining fixing solution	2 x 30 min
2.	Distilled or deionised water	5 x 5 min
3.	Sensitising solution	1 min
4.	Distilled or deionised water	2 x 1 min
5.	Silver solution	30 min
6.	Distilled or deionised water	2 x 1 min
7.	Developing solution	8 - 15 min
8.	Stopping solution	30 min
9.	Distilled or deionised water	3 x 5 min

REFERENCES

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

[2] Nour N., Mayer G., Mort J.S., Salvas A., Mbikay M., Morrison C.J., Overall C.M., Seidah N.G., The cysteine-rich domain of the secreted proprotein convertases PC5A and PACE4 functions as a cell surface anchor and interacts with tissue inhibitors of metalloproteinases, Mol Biol Cell 16 (2005) 5215-5226.

V. GENERAL DISCUSSION

V.1. OVERVIEW OF MANUSCRIPTS

The explosion of the world's population at an alarming rate (Kerr, 1995; Naz, 1996; Frayne and Hall, 1999), and the dissatisfaction of women with hormonal contraception, which eventually leads to discontinuation of usage (Schrager, 2002; Weber and Dohle, 2003), have called for the study of new contraceptive targets to curb the resulting unwanted pregnancies and abortions (Weber and Dohle, 2003); PCSK4 is one of such targets.

Though PCSK4 is expressed in the brain (Nelsen *et al.*, 2005), placenta (Qiu *et al.*, 2005), and ovaries (Tadros *et al.*, 2001), its major expression is in the testis and sperm (Nakayama *et al.*, 1992; Seidah *et al.*, 1992). This restricted expression led to the speculation of the enzyme playing an important role in fertilisation. Studies of mice lacking PCSK4 later confirmed that this enzyme is not only important in fertilisation, but also in early embryonic development (Mbikay *et al.*, 1997).

The biosynthesis of PCSK4 in testicular germ cells and its specific role(s) in fertilisation is/are not fully understood. Like all PCSKs, it is most probably biosynthesised as a secretory zymogen requiring autocatalytic activation, which then activates other secretory precursor protein(s) by limited endoproteolysis. Inferring from the infertility phenotype of male PCSK4-null mice (Mbikay *et al.*, 1997), these proproteins are probably involved in the functional maturation of sperm, sperm-egg interactions, and early embryo development. Understanding the cell biology of PCSK4 therefore and the identification of its physiological substrates might provide the basis for the design of contraceptive strategies targetting this enzyme or its substrates.

The work presented in this three-manuscript thesis seeks to partly fill up this knowledge gap using animal and cellular models. Each experiment described in this three-manuscript thesis was repeated at least three times. *Manuscript I* describes the *in vivo* subcellular localisation of PCSK4, as well as its requirement for normal sperm capacitation, acrosomal exocytosis, and sperm-zona binding. It demonstrates that PCSK4 is produced during spermatogenesis and is finally localised at the plasma membrane overlying the acrosome. Its absence causes sperm to undergo accelerated capacitation, become hypersensitive to ZP-induced acrosome reaction, less efficient in binding to egg's zona, and incapable of fertilising eggs *in vitro*. *Manuscript II* describes the effects of PCSK4 on *in vivo* post-translational modifications of sperm proteins; specifically, capacitation-induced protein tyrosine phosphorylation, and the proteolytic processing of precursors of sperm-egg ligands ADAM2 and ADAM3. It shows that, in the absence of PCSK4, capacitation-associated tyrosine phosphorylation of sperm proteins is enhanced, and that, proteolytic processing of the two ADAMs is quantitatively diminished. *Manuscript III* describes the *ex vivo* biosynthesis, maturation, and trafficking of PCSK4. It shows that, expressed in somatic cells, the bulk of PCSK4 zymogen is most likely retained in the ER where it interacts with GRP78/BiP, suggesting that its efficient activation in germ cells is facilitated by co-factors not found in somatic cells.

In the ensuing sections, I will first identify the strengths and limitations of our methodological approach. I will then discuss in an integrated fashion, the relevance of our findings for our general understanding of the roles of PCSK4 in cellular and physiological events leading to sperm acquisition of fertilisation competence. I will finally examine the implications of these findings for the long-term development of novel contraceptives.

V.2. LIMITATIONS OF MATERIALS, METHODS, AND ANALYSES

V.2.1. Antibodies

Since most of our results were generated using antibodies, a few concerns need to be addressed. A positive immunoreactivity does not always indicate a positive recognition of a protein or peptide of interest, as antibodies can cross-react with other molecules with similar epitopes. For example, an affinity-purified antiserum raised against human IGF-1B has been reported to be highly specific also for haemoglobin (Quinn *et al.*, 1996). To eliminate this concern, we raised and used different antibodies against PCSK4 (see Supplemental Table 2; p. 199). The most specific antibodies detected PCSK4 in WT sperm, but not in PCSK4-null sperm; in extracts from PCSK4-tansfected cells but not in those of untransfected cells or those transfected with the empty vector.

V.2.2. Molecular weight standards

Protein weight standards are also a matter of concern as markers supposedly of the same molecular weight from different companies when put side by side, most often, migrated with different electrophoretic mobilites in the same SDS-polyacrylamide gel. Though manufacturers claim protein standards are tested for proper mobility, thereby providing reliable control for gel-to-gel variability, we observed that this was not always the case. For non-radioactive gels, we used either SeeBlue Plus2 Pre-Stained Standard (Invitrogen) or Fermentas PageRuler Protein Ladder, and confirm that they do not always migrate at the same rate in an SDS-polyacrylamide gel much though, according to the manufacturers, they have the same molecular weights. The situation was even more apparent when Rainbow^{TM}

[^{14}C]Methylated protein molecular weight marker from Amersham was used in radioactive gels.

V.2.3. Semi-quantitative densitometry

The accuracy of comparative densitometric analysis of signals on X-ray film can be affected by the degree of exposure of the film. In all my analyses, I made several X-ray film exposures to insure that the signals were within the dynamic range of the densitometer; in this case, using Syngene's ChemiGenius Imaging System which is equipped with an extended dynamic range of up to 65 000.

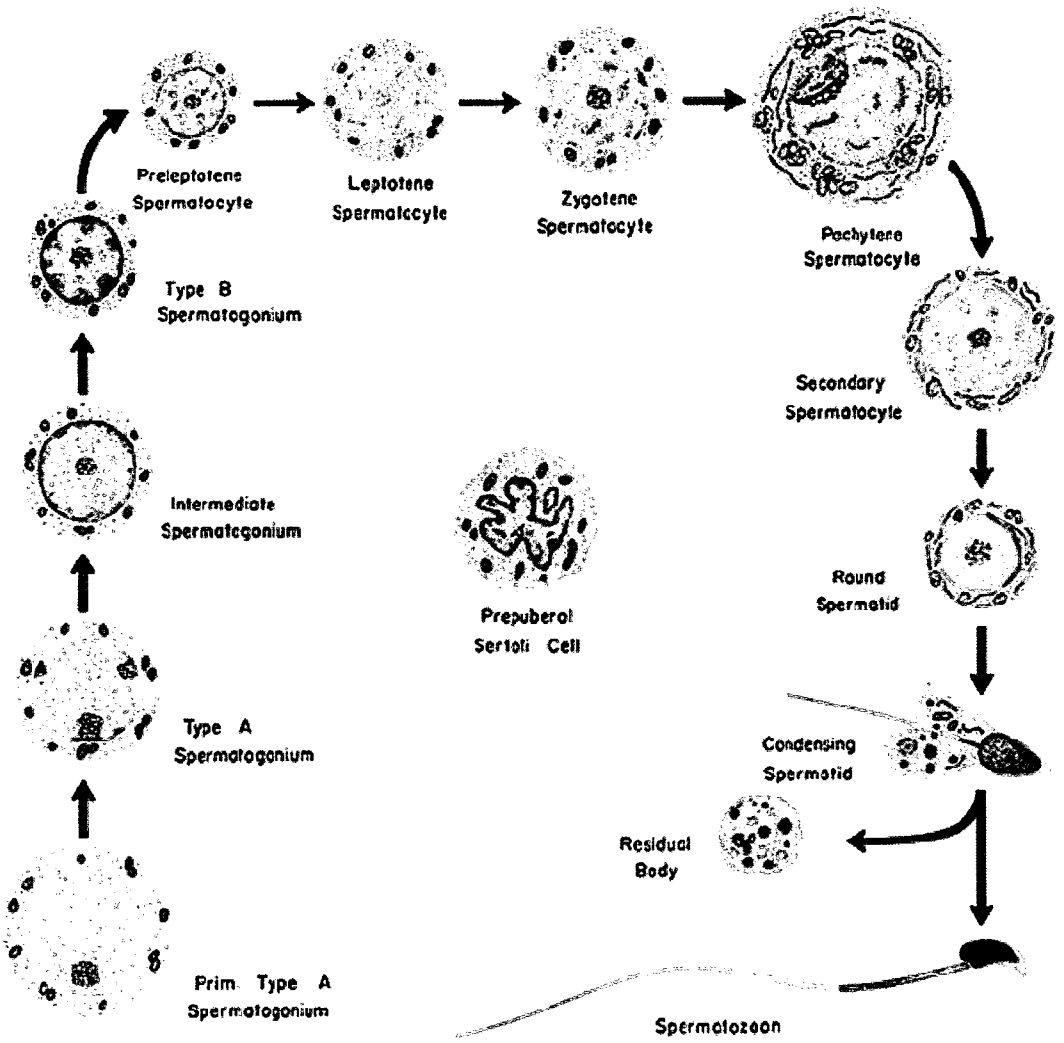
V.3. CONTRIBUTION OF PCSK4 TO SPERM FERTILISATION COMPETENCE

V.3.1. PCSK4 is expressed in testicular germ cells

Through continuous cellular differentiation, mammalian testicular germ cells (TGCs) differentiate into testicular sperm in a process called spermatogenesis. This process consists of spermatogonial renewal and proliferation, meiosis, and spermiogenesis (Fig. 1) (Bellvé *et al.*, 1977). It has initially been shown that *Pcsk4* transcripts are predominantly expressed in testicular germ cells, in spermatocytes, and in round spermatids in particular (Nakayama *et al.*, 1992; Seidah *et al.*, 1992). In *Manuscript I*, we show that the protein in its mature and presumably active form is found in these germ cell types, as well as in elongated spermatids and testicular sperm.

Specific proteins are expressed at different stages of the TGC differentiation. *Pcsk4* and *Pacap* transcripts, for example, appear on postnatal day 16 (p16) and p17 in mice,

Figure 1. *Schematic diagram of spermatogenesis in the prepuberal and adult mouse testis showing the relative volumes and characteristic morphology of the respective cell types.* This complex process occurs in three phases: the mitotic proliferation of spermatogonia (ascending axis); meiosis with its prolonged meiotic prophase (horizontal axis) and two reduction divisions which yield 2x secondary spermatocytes and then 4x haploid round spermatids; and spermiogenesis (descending axis) which culminates in the formation of spermatozoa. The cell types shown can be isolated by sedimentation velocity at unit gravity, based on their different cross-sectional areas, and temporal appearance during development. Thus, Sertoli cells and primitive type A spermatogonia can be isolated from mice at day 6 after birth, type A and type B spermatogonia at day 8, preleptotene, leptotene, zygotene, and pachytene spermatocytes from day 17, and pachytene spermatocytes, spermatids, and residual bodies from adult animals. Adapted from (Bellvé *et al.*, 1977; Bellvé, 1993).


Preleptotene
Spermatocyte
Leptotene
Spermatocyte
Zygotene
Spermatocyte
Pachytene
Spermatocyte
Type B
Spermatogonium
Secondary
Spermatocyte
Intermediate
Spermatogonium
Prepuberal
Sertoli Cell
Round
Spermatid
Type A
Spermatogonium
Condensing
Spermatid
Residual
Body
Prim. Type A
Spermatogonium
Spermatozoon

respectively (Tadros *et al.*, 2001; Li *et al.*, 2004); it is around this same period that TGCs differentiate into pachytene (primary) spermatocytes (Bellvé, 1993). Both PCSK4 and PACAP are also expressed in spermatocytes and spermatids (Shioda *et al.*, 1994; Gyamera-Acheampong *et al.*, 2006). Consistent with this co-localisation, ProPACAP was confirmed to be an obligatory physiological substrate for PCSK4 in TGCs (Li *et al.*, 1998; Li *et al.*, 2000a; Li *et al.*, 2000b).

Based on spatial and temporal co-expression (Forsbach and Heinlein, 1998; Kim *et al.*, 2003), proADAM1 and proADAM3 represent other potential PCSK4 substrates in TGCs. ProADAM1 processing in the testis of PCSK4-null mice could not be examined for lack of suitable antibodies. In *Manuscript II*, we show that the processing of ProADAM3 is not impaired; suggesting that it is not dependent on PCSK4, or is mediated by another PCSK or through a PCSK-unrelated protease.

V.3.2. PCSK4 is localised at the plasma membrane overlying the acrosome

After the differentiation processes, testicular sperm move out of the testis and continues its maturation in a long storage tube called the epididymis. The epididymis consists of caput (head), corpus (body), and cauda (tail) regions. In the epididymis, many sperm plasma membrane proteins undergo post-translational modification, including proteolytic processing, and multiple protein-protein interactions (Cooper, 2007). In *Manuscript I*, we show that PCSK4 is located on the surface of cauda epididymal sperm, specifically on the plasma membrane overlying the acrosome.

Because mouse and rat PCSK4 sequences do not contain a recognisable transmembrane domain, we investigated the nature of the association. In *Manuscript III*, we

show that the association is through hydrophobic interactions to water-insoluble and detergent-soluble components of the plasma membrane overlying the acrosome, and this association can be that can be disrupted only by molar concentrations of urea. The PCSK4 domain and the sperm components involved in these interactions are unknown.

Sperm surface PCSK4 may also be involved in proteolytic events associated with sperm epididymal transit. One such event is the conversion of the 103-kDa proADAM2 to its 50-kDa form. In *Manuscript II*, we show that this conversion is unimpaired in PCSK4-null sperm, suggesting that it is either not dependent on PCSK4, could be mediated by another PCSK, or is mediated by PCSK-unrelated protease. We also show that co-transduction of PCSK4 and ADAM2 in HEK293 cells supports the possibility of PCSK-mediated processing of pro-ADAM2. The alternate PCSKs could either be PCSK7, which is also found in epididymal sperm (our unpublished data), or PCSK3 (furin) shed by epididymal epithelial cells (Thimon *et al.*, 2006).

In this context, it is worth noting that the spatial and temporal links between PCSK4 and its substrates are broken as sperm differentiate and mature in the various gonadal compartments. For example, PACAP, the confirmed physiological substrate for PCSK4 in TGCs, is not expressed in mature spermatids, testicular sperm, nor epididymal sperm (Shioda *et al.*, 1994; Yanaihara *et al.*, 1998).

As will be discussed in Section V.5.3., localisation of PCSK4 to the surface of the plasma membrane overlying the acrosome makes it an accessible target for novel contraceptive development.

V.3.3. Lack of PCSK4 accelerates capacitation

Though epididymal transit confers on sperm the ability to move progressively, epididymal sperm are still fertilisation-incompetent, but after residing in the female genital tract for a finite period of time, they gain the ability to bind to eggs and fertilise (Austin, 1952). The physiological and biochemical changes which includes reorganisation of membrane proteins, metabolism of membrane phospholipids, reduction in membrane cholesterol levels, and hyperactivated motility (Yanagimachi, 1994); that confer on the sperm, the ability to fertilise, are termed Capacitation (Chang, 1951; Austin, 1952; Chang, 1955).

It was initially reported that PCSK4-null sperm from mice of a 129Sv/B6 mixed genetic background have reduced ability to fertilise eggs, and eggs fertilised by such sperm failed to grow to the blastocyst stage (Mbikay *et al.*, 1997). We have shown that in a pure B6 genetic background, the mutant sperm possesses no fertilising ability (Gyamera-Acheampong *et al.*, 2006). As capacitation confers on sperm the ability to fertilise, it was important to examine whether the fertilisation incompetence of PCSK4-null sperm emanates from altered capacitation. In *Manuscript II*, we show that these mutant sperm capacitate at a much faster rate relative to wildtype sperm. The reasons for this accelerated capacitation are unclear. It has been reported that the release of decapacitation factors (DFs) like plasma membrane fatty acid binding protein, cysteine-rich secretory protein 1 (CRISP1), phosphatidylethanolamine binding protein 1 (PBP1), and DF10 (Nixon *et al.*, 2006) from epididymal sperm surface during capacitation enables sperm to become fertilisation competent (Fraser, 1984; Fraser *et al.*, 1990). It remains to be determined whether lack of the PCSK4 causes sperm to have less or no active DFs at the surface, or whether they have normal levels of DFs but release them at a faster rate during capacitation. In the absence of a

mechanistic explanation, it can be speculated that, directly or indirectly, PCSK4 controls the cascade of events leading to progressive capacitation of sperm.

Since tyrosine phosphorylation is one of the biochemical modifications that occur during capacitation, we investigated whether this process is altered in PCSK4-null sperm. In *Manuscript II*, we show that the mutant sperm are hyper-tyrosine phosphorylated during capacitation; consistent with their faster capacitation rate. We also show that this phenomenon is mediated through the cAMP/PKA pathway: cAMP activates PKA which in turn phosphorylates protein tyrosine kinases. From SDS-PAGE gel slices containing hyperphosphorylated migrated proteins; we identified, through mass-spectrometry, proteins known to be subjected to capacitation-induced phosphorylation; they were the same in PCSK4-null and WT sperm. However, this approach could not decipher which of these proteins is/are susceptible to hyperphosphorylation in the PCSK4-null sperm. It is interesting to note that, although PCSK4 is located on the surface of the plasma membrane overlying the acrosome, protein tyrosine phosphorylation mostly occurs in the sperm tail; suggesting that the role of this enzyme in this event is preparatory rather than concurrent. It could be speculated that lack of PCSK4 leads to a disturbance in the balance of protein tyrosine kinase and phosphatase activities in capacitating sperm, ultimately altering signal transduction and sperm motility. This disturbance could also result in altered levels or activity of adenylyl cyclase/cAMP-modulating sperm proteins, such as Fertilisation Promoting Peptide and t-Complex 11 (TCP-11) (Adeoya-Osiguwa *et al.*, 1998), or CRISP 1 whose deficiency leads to reduced sperm protein tyrosine phosphorylation (Da Ros *et al.*, 2008).

V.3.4. PCSK4 plays no role in sperm penetration of egg cumulus mass

Capacitation results in altered patterns of sperm motility (Primakoff and Myles, 2002), as well as sperm penetration of nearly 3000 cumulus cells surrounding an egg through the assistance of hyaluronidases such as PH-20 (see Fig. 5; p. 26) (Salustri *et al.*, 1992; Primakoff and Myles, 2002). PH-20 is a GPI anchored surface hyaluronidase present on sperm surface which hydrolyses hyaluronic acid, a polymer consisting of repeating disaccharides units of D-glucuronic acid and *N*-acetyl-D-glucosamine, present in the cumulus mass (Lin *et al.*, 1994). It should however be noted that, sperm lacking PH-20 has been reported to be able to pass through the cumulus mass and fertilise the egg (Baba *et al.*, 2002); suggesting that the penetration of cumulus mass is either not carried out by PH-20 alone or is carried out through redundancy with other hyaluronidases. Lin *et al.* successfully blocked the crossing of cumulus mass by incubating acrosome intact sperm with anti-hyaluronidase antibody (Lin *et al.*, 1994). In *Manuscript I*, we show that cumulus penetration by PCSK4-null sperm is normal, excluding a role of the enzyme in this process.

V.3.5. Lack of PCSK4 reduces sperm binding to egg ZP

ZP is an egg coat consisting of three glycoproteins: ZP1, ZP2, ZP3 (Yanagimachi, 1994). It serves to protect the early embryo from the contractions of the oviductal musculature (Nichols and Gardner, 1989). It is the last physical barrier sperm traverse before gaining access to the egg plasma membrane (see Fig. 5; p. 26). Before penetration, capacitated sperm binds firmly to the zona surface (Bleil and Wassarman, 1983) through interactions between zona and sperm surface proteins (Gwatkin and Williams, 1977).

Freshly ejaculated sperm or uncapacitated epididymal sperm can bind to zonae from homologous or heterologous species (Swenson and Dunbar, 1982; Yanagimachi, 1994), but the attachment is weak and usually not species-specific. However, the attachment between a capacitated sperm and the zona is not easily dislodged, and it is species-specific (Inoue and Wolf, 1975; Saling *et al.*, 1978; Yanagimachi, 1994).

In *Manuscript I*, we show that capacitated PCSK4-null sperm has reduced binding to the zona; suggesting they either bind weakly to the zona or carry fewer zona ligands on their surfaces. Two such ligands are ADAM2 (fertilin β) and ADAM3 (cyritestin). They result from limited proteolysis of larger secretory precursors (Blobel *et al.*, 1990; Linder *et al.*, 1995; Lum and Blobel, 1997; Shamsadin *et al.*, 1999). Proteolytic processing of ADAM2 has been reported to be correlated with the acquisition of sperm fertilisation competence (Blobel *et al.*, 1990). ADAM2 enables sperm to migrate from the uterus into the oviduct, bind to the egg's ZP, and attach to the egg's membrane (Cho *et al.*, 1998). In contrast, ADAM3 is not required for migration into the oviduct but is required for the binding to the egg's ZP and plasma membrane (Shamsadin *et al.*, 1999; Nishimura *et al.*, 2001). Genetically modified male mice lacking ADAM2 or ADAM3 are infertile (Cho *et al.*, 1998; Shamsadin *et al.*, 1999; Nishimura *et al.*, 2001).

Near the putative processing sites within proADAM2 and 3 sequences are found motifs recognisable by kexin-like PCSK. Their presence suggests that the processing could be mediated by this PCSK subfamily. ProADAM3 processing is a testicular event (Linder *et al.*, 1995); that of proADAM2 is an epididymal event (Blobel *et al.*, 1990). In *Manuscript II*, we show that neither event is significantly impaired in PCSK4-null sperm. We also report that capacitation is accompanied by further conversion of a ~46-kDa form of ADAM2 to a

~27 kDa; a process that can be blocked by a general PCSK inhibitor. Furthermore, we show that these enzymes could process proADAM2 in HEK293 cells co-transfected with expression vectors. Collectively, these observations suggest that proADAM2 processing of enzymes in TGCs and sperm are PCSK-like and redundant. The two PCSKs found in these cells are PCSK4 and PCSK7. However, unlike PCSK4-null mice, PCSK7-null mice are fertile (Scamuffa *et al.*, 2006). This phenotypic difference indicates that PCSK7 may activate some but not all substrates in common with PCSK4; PCSK4-dependent substrates may be uniquely relevant for fertility.

V.3.6. Lack of PCSK4 renders sperm hypersensitive to ZP-induced AR

Capacitation also confers on the sperm the ability to undergo regulated acrosome reaction (Primakoff and Myles, 2007): the exocytosis of their acrosomal contents following binding to the ZP (Yanagimachi, 1994; Primakoff and Myles, 2002) (see Fig. 5; p. 26). The released acrosomal content contains several hydrolases, including hyaluronidase, arylsulfatase, acid and alkaline phosphatases, glycosidases, phospholipase A, neuraminidase, metalloendoprotease, and proteases (Yanagimachi, 1994). Through the action of these hydrolases, the ZP is perforated allowing the sperm head to enter into the perivitelline space to initiate sperm-egg fusion (see Fig. 5; p. 26) (Primakoff and Myles, 2002).

Acrosome reaction results in the relocalisation of membrane proteins such as PH-20 (Myles *et al.*, 1987) and Izumo (Inoue *et al.*, 2005) into other membrane regions. Additional alterations of sperm membrane molecules following acrosomal exocytosis may result in the exposure of some proteins that can bind to the sperm plasma membrane and/or alter it by enzymatic activity (e.g., proteases and glycosidases) (Primakoff and Myles, 2007).

Acrosome reaction can be studied *in vitro* using capacitated sperm and solubilised zona. Such zona induces acrosome reaction faster (Florman and Storey, 1982), more efficiently (Bleil and Wassarman, 1983), and in less species specific manner (Moller and Wassarman, 1989) than "native zona".

In *Manuscript I*, we show that, *in vitro*, PCSK4-null sperm are more sensitive to solubilised zona-induced acrosomal exocytosis than WT sperm. The increased responsiveness may be mechanistically linked to a stimulation of adenylate cyclase/PKA as reflected by the accelerated tyrosine phosphorylation of sperm proteins during capacitation.

Members of the PCSK family have been implicated in proZP proteolytic processing at the cell surface (Kiefer and Saling, 2002) and whether they, including PCSK4, participates in ZP hydrolysis following acrosome reaction remains to be determined.

Many cell to cell interactions are mediated through the RGD sequence motif in some proteins (Plow *et al.*, 1985; Yamada and Kennedy, 1985; Ruoslahti and Pierschbacher, 1986). PCSK4 possesses an RGD motif in its P-domain; this suggests that PCSK4 might interact with membrane proteins such as egg surface integrins.

V.4. BIOSYNTHESIS, MATURATION, AND TRANSPORT OF HUMAN PCSK4

V.4.1. Background

PCSKs are initially biosynthesised as a zymogen and undergo autocatalytic activation in the ER (except PCSK2); this results in a heterodimer between the prosegment and the remaining part of the protein (Seidah *et al.*, 2008). Synthetic peptides derived from the prosegments of PCSK1 and PCSK3 are potent inhibitors of these enzymes (Basak and Lazure, 2003). The prosegment exerts potent inhibitory effects on the enzyme until it is

released. PCSK5 and PCSK6 are activated at the cell surface where they are tethered to heparan sulfate proteoglycans (Mayer *et al.*, 2008). This activation pathway is unique and differs from that of PCSK3 and PCSK7, which are activated in the trans-Golgi network. It also differs from PCSK1 and PCSK2; they are activated in dense core secretory granules (Seidah *et al.*, 2008). In all these activation cases, a cleavage at a secondary cleavage site within the prosegment results in its release. How PCSK4 is synthesised, transported, and activated has never been investigated previously. Elucidating this process may lead to a better understanding of the cell biology of this enzyme and its preferential expression in TGCs.

V.4.2. PCSK4 is inefficiently matured and transported in somatic cells

In *Manuscript III*, we attempted to address the biosynthesis, maturation, and transport of PCSK4 with *ex vivo* experiments. We show that, when transduced in HEK293 cells, rat and human PCSK4 remain predominantly as intracellular zymogens, presumably retained in the ER, where it interacts with GRP78/BiP. It is possible that this interaction with BiP contributes to the retention; this chaperone has been shown to act in a similar manner with a mutant low-density lipoprotein (Jorgensen *et al.*, 2000). The interaction could be promoted by the inadequate folding of the transduced glycoprotein. Proper folding of glycoproteins such as PCSK4 with a C-terminal glycan is facilitated by protein disulfide isomerases (PDIs) and BiP-like chaperones (Molinari and Helenius, 2000). Spermiogenesis is associated with the haploid-cell specific expression of the annexin-related calmegin (CLGN) and the PDI-like of testis (PDILT) (Watanabe *et al.*, 1994; van Lith *et al.*, 2007). It is possible the CLGN-PDILT complexes are more efficient than BiP-PDI complexes at promoting the proper

folding of newly biosynthesised PCSK4. Inactivation of the *Clgn* gene in mouse leads to abnormal trafficking of a variety of spermiogenesis-induced gene products including ADAM1, ADAM2, ADAM3 and angiotensin converting enzyme (Ikawa *et al.*, 2001; Yamaguchi *et al.*, 2006). It would be interesting to determine whether PCSK4 transport is also altered in haploid TGCs of these mice. Male CLGN-null mice are infertile (Ikawa *et al.*, 1997).

We were intrigued by the fact that PCSK3, the closest homologue of PCSK4, is efficiently matured and transported to the cell surface when transduced in HEK293 cells (Molloy *et al.*, 1994; Molloy *et al.*, 1999). We therefore examined whether, besides misfolding, specific motifs present in the hPCSK4 sequence could be responsible for the retention in the ER. Alignment of the human PCSK3 and PCSK4 indicate that the sequence of these two convertases mostly diverge in the signal peptide and CT domain (amino acid homology - signal peptide: 16%; prodomain: 41%; catalytic domain: 68%; P-domain: 40%; CT: 16%). Truncation of the hPCSK4 sequence up to transmembrane domain or the P-domain did not abrogate the retention or promote proteolytic activation of the zymogen. Most interestingly, when the propeptide of this enzyme was expressed as a distinct secretory entity, it was also retained intracellularly. Using the Eukaryotic Linear Motif (ELM, http://ca.expasy.org/tools/) algorithm to search for functional sites not shared by the prodomains of human PCSK3 and PCSK4, we identified a PDZ ligand tetrapeptide A/V-R-E-L that is highly conserved among PCSK4 orthologues (Fig. 2) but absent in its paralogues (Fig. 3). PDZ is a domain with a name derived from the first letters of three proteins: **P**ostsynaptic Density Protein (PSD95), **D**rosophila disc large tumour suppressor (DlgA), and **Z**onula Occludens-1 protein (ZO-1); the first three proteins discovered to share the domain. PDZ domain proteins are found in the ER (Ma *et al.*, 2006), and PDZ motifs are modular

protein–protein interaction domains, consisting of 80–120 amino acid residues, whose function appears to be the direction of intracellular proteins to multiprotein complexes in subcellular compartments. They bind to ligand motifs sometimes located in the internal region, but mostly located at the C-terminus of the interacting protein (Hung and Sheng, 2002). PDZ ligand masking has been proposed to be a mechanism of releasing ER-retained proteins and allowing their transport to downstream secretory compartments (Michelsen *et al.*, 2005). Several PDZ domain proteins are expressed in mammalian testis and localised to the apical acrosomal region and thus may organise signalling molecules mediating the various reactions leading to fertilisation. Whether PDZ ligand motifs contribute to the ER retention of proPCSK4 in somatic cells could eventually be investigated by prodomain swapping and site-directed mutagenesis.

Figure 2. *Prosegment sequence alignment of some orthologues of PCSK4.* The conserved PDZ ligand tetrapeptide, A/V-E-R-L, present in all PCSK4 orthologues shown in red.

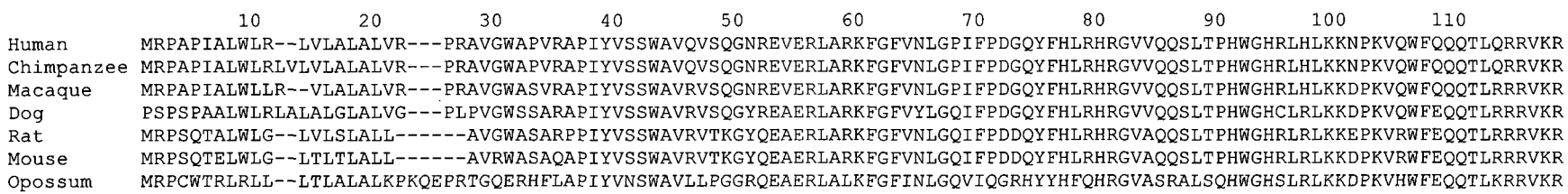
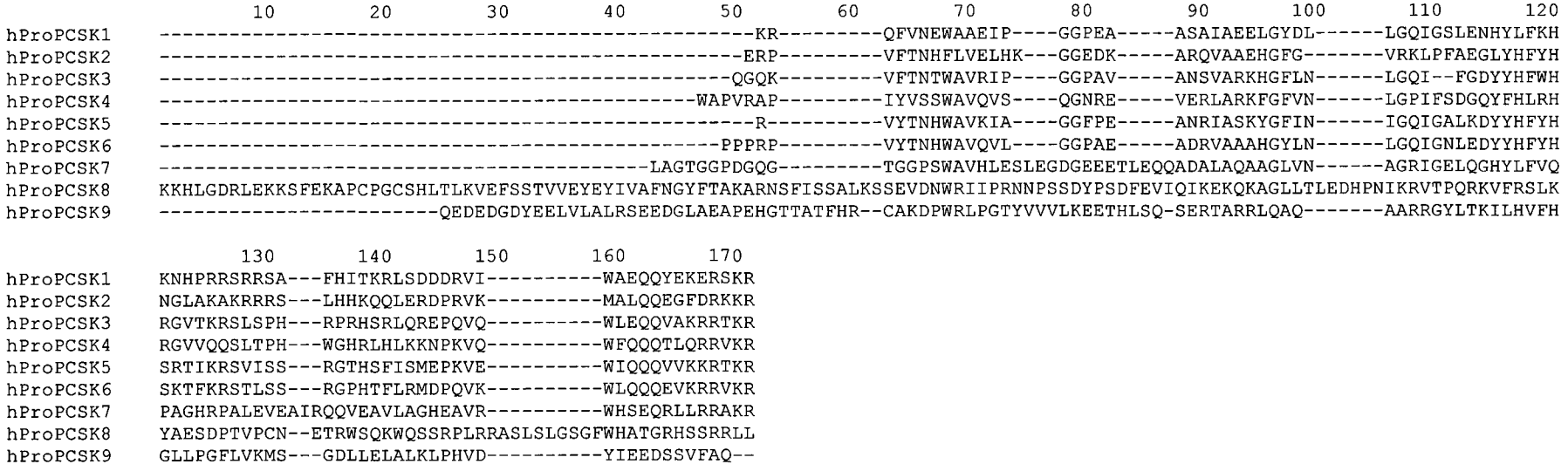

10 20 30 40 50 60 70 80 90 100 110
Human MRPAPIALWLR--LVLALALVR---PRAVGWAPVRAPIYVSSWAVQVSQGNREVERLARKFGFVNLGPIFPDGQYFHLRHRGVVQQSLTPHWGHRLHLKKNPKVQWFQQQTLQRRVKR
Chimpanzee MRPAPIALWLRLVLVLALALVR---PRAVGWAPVRAPIYVSSWAVQVSQGNREVERLARKFGFVNLGPIFPDGQYFHLRHRGVVQQSLTPHWGHRLHLKKNPKVQWFQQQTLQRRVKR
Macaque MRPAPIALWLLR--VLALALVR---PRAVGWASVRAPIYVSSWAVRVSQGNREVERLARKFGFVNLGPIFPDGQYFHLRHRGVVQQSLTPHWGHRLHLKKDPKVQWFQQQTLRRRVKR
Dog PSPSPAALWLRLALALGLALVG---PLPVGWSSARAPIYVSSWAVRVSQGYREAERLARKFGFVYLGQIFPDGQYFHLRHRGVVQQSLTPHWGHCLRLKKDPKVQWFEQQTLRRRVKR
Rat MRPSQTALWLG--LVLSLALL------AVGWASARPPIYVSSWAVRVTKGYQEAERLARKFGFVNLGQIFPDDQYFHLRHRGVAQQSLTPHWGHRLRLKKEPKVRWFEQQTLRRRVKR
Mouse MRPSQTELWLG--LTLTLALL------AVRWASAQAPIYVSSWAVRVTKGYQEAERLARKFGFVNLGQIFPDDQYFHLRHRGVAQQSLTPHWGHRLRLKKDPKVRWFEQQTLRRRVKR
Opossum MRPCWTRLRLL--LTLALALKPKQEPRTGQERHFLAPIYVNSWAVLLPGGRQEAERLALKFGFINLGQVIQGRHYYHFQHRGVASRALSQHWGHSLRLKKDPKVHWFEQQTLKRRVKR

Figure 3. *Sequence alignment of prosegments of PCSKs.* The PDZ ligand tetrapeptide, V-E-R-L (in red), is present only in human PCSK4 and not in the other paralogues.


10 20 30 40 50 60 70 80 90 100 110 120
hProPCSK1 ----------KR---------QFVNEWAAEIP----GGPEA-----ASAIAEELGYDL------LGQIGSLENHYLFKH
hProPCSK2 ----------ERP---------VFTNHFLVELHK---GGEDK-----ARQVAAEHGFG-------VRKLPFAEGLYHFYH
hProPCSK3 ----------QGQK---------VFTNTWAVRIP----GGPAV-----ANSVARKHGFLN------LGQI--FGDYYHFWH
hProPCSK4 ----------WAPVRAP---------IYVSSWAVQVS----QGNRE-----VERLARKFGFVN------LGPIFSDGQYFHLRH
hProPCSK5 ----------R----------VYTNHWAVKIA----GGFPE-----ANRIASKYGFIN------IGQIGALKDYYHFYH
hProPCSK6 ----------PPPRP---------VYTNHWAVQVL----GGPAE-----ADRVAAAHGYLN------LGQIGNLEDYYHFYH
hProPCSK7 ----------LAGTGGPDGQG---------TGGPSWAVHLESLEGDGEEETLEQQADALAQAAGLVN------AGRIGELQGHYLFVQ
hProPCSK8 KKHLGDRLEKKSFEKAPCPGCSHLTLKVEFSSTVVEYEYIVAFNGYFTAKARNSFISSALKSSEVDNWRIIPRNNPSSDYPSDFEVIQIKEKQKAGLLTLEDHPNIKRVTPQRKVFRSLK
hProPCSK9 ----------QEDEDGDYEELVLALRSEEDGLAEAPEHGTTATFHR--CAKDPWRLPGTYVVVLKEETHLSQ-SERTARRLQAQ-------AARRGYLTKILHVFH
130 140 150 160 170
hProPCSK1 KNHPRRSRRSA---FHITKRLSDDDRVI----------WAEQQYEKERSKR
hProPCSK2 NGLAKAKRRRS---LHHKQQLERDPRVK----------MALQQEGFDRKKR
hProPCSK3 RGVTKRSLSPH---RPRHSRLQREPQVQ----------WLEQQVAKRRTKR
hProPCSK4 RGVVQQSLTPH---WGHRLHLKKNPKVQ----------WFQQQTLQRRVKR
hProPCSK5 SRTIKRSVISS---RGTHSFISMEPKVE----------WIQQQVVKKRTKR
hProPCSK6 SKTFKRSTLSS---RGPHTFLRMDPQVK----------WLQQQEVKRRVKR
hProPCSK7 PAGHRPALEVEAIRQQVEAVLAGHEAVR----------WHSEQRLLRRAKR
hProPCSK8 YAESDPTVPCN--ETRWSQKWQSSRPLRRASLSLGSGFWHATGRHSSRRLL
hProPCSK9 GLLPGFLVKMS---GDLLELALKLPHVD----------YIEEDSSVFAQ--

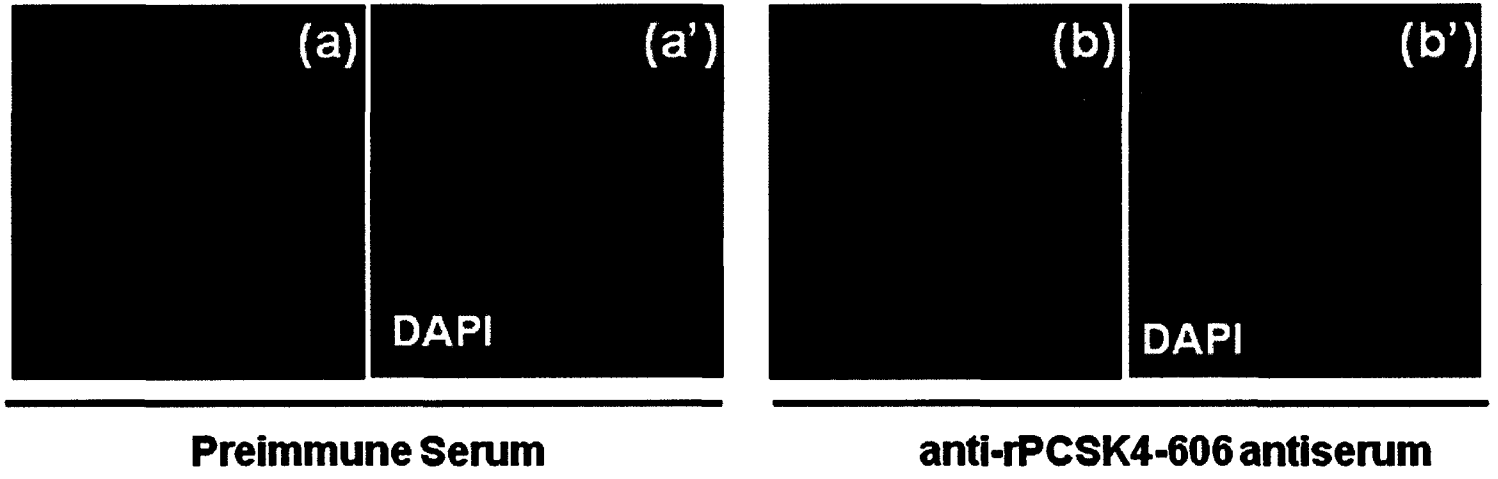
V.4.3. Detection of enzymatic activity of transduced PCSK4

We show in *Manuscript II* the processing of proADAM2 when co-transduced with PCSK4 at a substrate/enzyme ratio of 100. The processing was not evident when the transduction was carried out with proADAM2 alone. A similar situation was observed with the transduction of proIGF2 in *Manuscript III*. ProIGF2 carries a number of basic motifs recognisable by PCSKs (see Figure 10A; p. 175). Some of these sites are cleaved by PCSK1, 3, 4, or 7 (Duguay *et al.*, 1998; Qiu *et al.*, 2005); but in a human placental cell line, cleavage after the $KSER^{68}$ motif leading to the production of fully matured IGF2 is exclusively mediated by PCSK4 (Qiu *et al.*, 2005). PCSK4 cleaves after a P1 Arg with a P4 Lys better than other Kexin-like convertases (Basak *et al.*, 2004; Basak *et al.*, 2008). Also, insertion of the KSER motif into the reactive site loop (RSL) of hPCIN resulted in a recombinant serpin ($hPCIN^{KSER}$) capable of inhibiting hPCSK4 enzymatic activity. ProIGF2 was co-transduced with hPCSK4 in the presence of hPCIN or $hPCIN^{KSER}$. We show in *Manuscript III* that expression of proIGF2 alone results in the secretion proIGF2 and two other smaller peptides (see Figure 10B; p. 175); however, co-transduction with hPCSK4 resulted in the downregulation of the precursor and complete disappearance of the smaller peptides (see Figure 10B; p. 175). These hPCSK4-induced changes were maintained in the presence of hPCIN (see Figure 10B; p. 175), but were reversed in the presence of $hPCIN^{KSER}$. These results taken together go to buttress the fact that the tranduced PCSK4 was enzymatically active.

V.5. IMPLICATIONS OF PCSK4 FOR FERTILITY

The body of evidence discussed above strongly indicates that PCSK4 is important for male fertility in mouse. The high conservation of its sequence among tetrapods, the testicular abundance of its mRNA in mammals, and its presence on human sperm plasma membrane overlying the acrosome (Fig. 4) suggest that it may be critical for human fertility too. In the western world, 1 in 6 couples experiences fertility difficulties. Half of these cases are due to the male-factor infertility (MFI). A third of MFI cases are associated with chromosomal abnormalities; the remaining two thirds are of unknown origin (Bhasin *et al.*, 1994; Brugh *et al.*, 2003). Could PCSK4 deficiency contribute to some of the idiopathic infertilities, either because of failed expression, abnormal expression, or impaired activity? Could PCSK4 be a target for male contraception?

Figure 4. *PCSK4 in human sperm.* Percoll gradient-purified human intact sperm in suspension were treated with rabbit preimmune serum (a, a') or anti-rPCSK4-606 antibody (b, b'). After washing, they were fixed on microscope slides. The slides were stained with Alexa fluor 594 goat anti-rabbit IgG (a, b) and counterstained with the DAPI DNA dye (a', b'). The fluor and DAPI stains were visualised by fluorescence microscopy. PCSK4-specific immunoreaction was detected over the acrosome (b) above the nucleus (b').


(a)
(a')
DAPI
(b)
(b')
DAPI
Preimmune Serum
anti-rPCSK4-606 antiserum

V.5.1. PCSK4-based assays for sperm fertilising ability

A critical role of PCSK4 in human fertility would render unlikely intergenerational transmission of severely deleterious mutations in its gene. On the other hand, alteration in expression due to developmental or environmental injuries could be a cause of infertility in human. Such injuries could be reflected by abnormal transcription of the gene. A review of ESTs deposited in DNA databases indicate that, like in mouse, human *PCSK4* gene is transcribed in multiple mRNA isoforms resulting from exon skipping, optional exon insertion, and encoding PCSK4 isoforms with in-frame or frameshift insertions/deletions. Enzymatically, these isoforms could be active, inactive, or inhibitory. An assay for the overall PCSK4 activity per a given sperm number or per levels of immunoreactive PCSK4 may provide some indication of the fertilising ability of the sperm pool. This activity could be deduced from the total PCSK-like hydrolytic measured activity using a PCSK4-specific fluorogenic substrate minus the remaining activity in the presence of an excess of PCSK4-specific inhibitor. 7B2, a PCSK2-specific inhibitor, which also acts as a chaperone, has been used in a similar manner to measure the activity of this convertase in various mouse tissues (Li *et al.*, 2003).

Alternatively, PCR-based profiling of *Pcsk4* mRNA isoforms in ejaculated sperm could be used to assess the quality of PCSK4 gene expression. A number of reports of the last decade have shown that ejaculated human sperm contains a variety of mRNAs (Miller *et al.*, 1999; Ostermeier *et al.*, 2002; Grunewald *et al.*, 2005; Miller *et al.*, 2005; Ostermeier *et al.*, 2005a; Ostermeier *et al.*, 2005b; Miller and Ostermeier, 2006). This limited transcriptome is a remnant of transcriptional events that took place during spermatogenesis (Grunewald *et al.*, 2005). It is transmitted to the egg upon fertilisation and may be of

importance for early development. Because ejaculated sperm can be obtained with relative ease, changes in its RNA content and profile can be used to assess the fertility and the consequences of environmental toxicants on fertility (Ostermeier *et al.*, 2002; Miller and Ostermeier, 2006). As *PCSK4* transcripts can be detected in ejaculated human sperm, their splicing profile could be used as a marker of sperm quality.

In the context of artificial reproductive technology, it may be worth examining whether supplementation of purified recombinant PCSK4 to deficient sperm could improve their egg fertilisation ability *in vitro*.

V.5.2. PCSK4 as pharmacological contraceptive target?

The absence of overt spermatogenetic or steroidogenetic defects in male PCSK4-null mice suggests that therapeutic targeting of PCSK4 for contraceptive purposes in man may not be associated with the endocrinological pitfalls of hormonal contraception. Current efforts to develop PCSK-specific inhibitors take advantage of the properties of the prodomain as an intramolecular chaperone and inhibitor (Fugere and Day, 2002; Basak, 2005). A synthetic peptide based on the sequence overlapping the demarcation between the pro and the catalytic domains of PCSK4 has recently been shown to inhibit this enzyme at low micromolar concentrations (Basak *et al.*, 2008). However, prodomain-based inhibitors often exhibit broad specificity for members of the PCSK family (Fugere *et al.*, 2002). Restricting this specificity remains a major challenge. Additional challenges include the mode of administration of the inhibitor and its immunogenicity. Topical application in the female reproductive tract is a conceivable approach. Mucosal or humoral antibodies induced by this application may eventually counter the contraceptive efficacy of the inhibitor without

the snags. Success in this effort will depend on a better understanding of the biological functions of PCSK4 and its natural substrates.

V.5.3. PCSK4 as a target in immunocontraception

Immunocontraception is achieving contraception through the use of antibodies. Not all immunogens are ideal to illicit immunocontraception. Preferable contraceptive immunogens should among other things be sperm-specific to prevent cross-reactivates with immunogenic epitopes on somatic cell components, have a fertility-related function that can be inhibited by an antibody, be exposed on the surface of sperm to enable antibody recognition so as to affect sperm motility, be shown to inhibit fertility in animal models, and be able to inhibit prefertilisation events (Herr, 1996; Frayne and Hall, 1999).

Owing to these, recent attempts for developing reversible forms of non-hormonal contraception for males have focussed on targeting sperm surface proteins involved in or responsible for the functional maturation of sperm or sperm-egg interaction (Kerr, 1995; Herr, 1996; Naz, 1996), and one such protein is PCSK4.

PCSK4 plays a crucial role in fertilisation, however its specific role(s) is/are not fully understood. It is surmised that PCSK4 activate a variety of sperm/egg surface proteins, or its RGD motif within the P-domain mediates binding to egg integrins. PCSK4 is a protein on the sperm surface, and its lack results in sperm fertilisation incompetence (Mbikay *et al.*, 1997), uncontrolled sperm capacitation, precocious acrosome reaction, and inability of sperm to bind to zona. Based on these characteristics, one could speculate PCSK4 as a candidate for contraceptive immunogen. We show in *Manuscript I* that primary spermatocytes, round spermatids, and elongated spermatids are among TGCs expressing PCSK4; and that PCSK4

is located on the surface of cauda epididymal sperm, specifically on the plasma membrane overlying the acrosome. Work needs to be done to access the conceptive efficacy of using PCSK4 as immunogen, as well as finding out if a specific region or the whole protein could elicit the desired contraceptive response in experimental animals.

V.5.4. References

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V.I. CURRICULUM VITAE

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PUBLICATIONS

1. **Charles Gyamera-Acheampong** and Majambu Mbikay (2009). **Relevance of proprotein convertase subtilisin/kexin type 4 (PCSK4) in mammalian fertility (a review).** *Human Reproduction Update* **15(2)**:237-247

2. **Charles Gyamera-Acheampong,** Julian Vasilescu, Daniel Figeys, and Majambu Mbikay (2009). **PCSK4-null sperm display enhanced protein tyrosine phosphorylation and ADAM2 proteolytic processing during *in vitro* capacitation.** *Fertility and Sterility: In press.*

3. Mayne, J., Raymond, A., Chaplin, A., Cousins, M., Kaefer, N., **Gyamera-Acheampong, C.**, Seidah, N. G., Mbikay, M., Chretien, M., and Ooi, T. C. (2007). **Plasma PCSK9 levels correlate with cholesterol in men but not in women.** *Biochem Biophys Res Commun* **361**, 451-456.

4. **Gyamera-Acheampong C**, Tantibhedhyangkul J, Weerachatyanukul W, Tadros H, Xu H, van de Loo JW *et al* (2006). **Sperm from mice genetically deficient for the PCSK4 proteinase exhibit accelerated capacitation, precocious acrosome reaction, reduced binding to egg zona pellucida, and impaired fertilising ability.** *Biol Reprod* **74**:666-73.

5. Schmidt, G., Sirois, F., Anini, Y., Kauri, L. M., **Gyamera-Acheampong, C.**, Fleck, E., Scott, F. W., Chretien, M., and Mbikay, M. (2006). **Differences of pancreatic expression of 7B2 between C57BL/6J and C3H/HeJ mice and genetic polymorphisms at its locus (Sgne1).** *Diabetes* **55**, 452-459.

SUBMITTED MANUSCRIPT

Charles Gyamera-Acheampong, Francine Sirois, Nicholas J. Denis, Daniel Figeys, Majambu Mbikay. **ProPCSK4 is slowly matured to its active form in transfected somatic cells: evidence of interactions with BiP and of enzymatic activity.** *Revised and re-submitted to Biochimie on April 24, 2009.*

ABSTRACT AND POSTERS

1. **Gyamera-Acheampong *Charles***, Ndaya-Lumbala *Annie*, and Mbikay *Majambu.* **Effects of an engineered PCSK4 serpin and anti-PCSK4 antibodies on fertilisation *in vitro.*** *Graduate Poster Day,* BMI, University of Ottawa, Ottawa. Canada. May 2007.

2. **Gyamera-Acheampong *Charles*** and Mbikay *Majambu.* Ascertaining if the sperm cell protease PCSK4 is a good target for non-hormonal contraception. *39th Annual SSR Meeting.* Omaha, Nebraska, U.S.A. July 29 - August 1, 2006.

3. **C. Gyamera-Acheampong,** H. Tadros, F. Sirois, J. Tantibhedyangkal, W. Weerachatyanukul, N. Tanphaichitr, and M. Mbikay. **Proprotein Convertase Subtilisin-Kexin Type 4 (PCSK4) is located on the Acrosomal Plasma Membrane and its deficiency renders spermatozoa less efficient at egg-binding.** *Graduate Poster Day,* BMI, University of Ottawa, Ottawa. Canada. May 2005.

4. **C. Gyamera-Acheampong,** H. Tadros, F. Sirois, J. Tantibhedyangkal, W. Weerachatyanukul, N. Tanphaichitr, and M. Mbikay. **Proprotein Convertase 4 (PC4) is located on the Acrosomal Plasma Membrane and its deficiency renders spermatozoa less efficient at egg-binding.** *5th Annual OHRI Research Day,* Ottawa, Ottawa. Canada. November 2004.

5. **C. Gyamera-Acheampong,** H. Tadros, F. Sirois, C. St. Germain, J. Mayne, J. Hazelwood, F. Dong, J. Kelly, M.Chretien, and M. Mbikay. **Identification and Characterisation of Protein Alterations in PCSK4-deficient Sperm.** *22nd Annual Ottawa Reproductive Biology Workshop,* Ottawa. Canada. May 2004.

6. **Charles Gyamera-Acheampong,** Gunther Schmidt, Majambu Mbikay. **The Sperm Cell Protease PCSK4 as a Target in Contraceptive Development.** *Graduate Poster Day,* BMI, University of Ottawa, Ottawa. Canada. May 2003.

PRESENTATIONS

1. **Gyamera-Acheampong, C.** Ascertaining if the sperm cell protease PCSK4 is a good target for non-hormonal contraception. *Proprotein Convertase Group Seminar Series.* OHRI. Ottawa. January 24, 2007.

2. **Gyamera-Acheampong, C.** Precocious Capacitation and acrosome reaction in PCSK4-null sperm. *BMI Symposium Research Seminars.* University of Ottawa. Ottawa. Canada. February 22, 2006.

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10. **Gyamera-Acheampong, C.** Tyrosine Phosphorylation of HSP-90 During Mammalian Sperm Capacitation [Biology of Reproduction (2003) *69*: 1801–1807]. *Proprotein Convertase Group Journal Club Series.* March 17, 2004

11. **Gyamera-Acheampong, C.** Antibodies to Malaria Peptide Mimics Inhibit *Plasmodium falciparum* Invasion of Erythrocytes. [Infection and Immunity (2004) *72(2)*: 1126 - 1134]. *Proprotein Convertase Group Journal Club Series.* June 9, 2004.

SCHOLARSHIPS, FELLOWSHIPS, AND OTHER AWARDS

Name of Award	Value/YR (CAN$)	Level	Type	Location of Tenure	Period Held
Fellowship	7 000	Institutional	Academic	University of Ottawa	02/2008-07/2008
NSERC Fellowship	17 000	National	Academic	University of Ottawa	09/2002-01/2008
Most Convincing Student; Ph.D. Biochemistry Graduate Poster Competition	Laser Pointer	Institutional	Research	University of Ottawa	05/2007
Buroughs Wellcome Travel Award		International	Research	SSR, Omaha, Nebraska, USA	08/2006
2nd Prize; Ph.D. Biochemistry Graduate Poster Competition	75	Institutional	Research	University of Ottawa	05/2005
Norwegian Government Scholarship for Graduate Students from Developing Countries	13 000	International	Academic/ Research	University of Tromsø, Norway	08/1995-11/1998
Ghana Government Scholarship for postgraduate studies	7 000	National	Academic/ Research	University of Cape Coast, Ghana	10/1992-10/1993
Ghana Government Scholarship for undergraduate studies	5 000	National	Academic	University of Science and Technology, Ghana	10/1985-10/1989

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Available upon request