Identification and characterisation of novel protein kinase A Cβ splice variants expressed in NT2-N cells differentiated by retinoic acid

Cand. scient. thesis

by

Anja Camilla Vogeler Larsen

Department of Nutrition

Faculty of Medicine

UNIVERSITY OF OSLO

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Anja Camilla Vogeler Larsen

Abstract

Protein kinase A (PKA) is a holoenzyme consisting of a regulatory (R) subunit dimer and two catalytic (C) subunits. Humans contain at least four genes that express four isoforms of the C subunits, designated Cα, Cβ, Cγ and PrKX. Both the Cα and the Cβ genes express splice variants, all of which differ in the N-terminal part encoded by exon 1. It has previously been shown that retinoic acid (RA), which promotes differentiation of the human neuronal precursor cell line Ntera 2 (NT2) to form NT2-N nerve cells, also promotes the induction of a number of nerve cell specific Cβ splice variants which may be important for PKA-dependent regulation of nerve cell differentiation and function. In this thesis, we show that RA also induces 6 novel mRNA species from the Cβ gene, which lack the nucleotides transcribed from the exon 4. Identification of Cβ forms lacking the exon 4 sequence was also confirmed in human brain mRNA. This was concluded using a method that was developed in order to specifically detect low levels of mRNA species encoding Cβ variants lacking the exon 4. When using this method, we also demonstrated that alternative splicing of the exon 4 probably does not occur in human peripheral blood leukocytes, despite the fact that they express the Cβ1 and Cβ2 variants. This implies that alternative splicing of the Cβ exon 4 may only take place in nerve cells. The novel splice variants displayed no *in vitro* catalytic activity and did not induce the expression of a CRE-regulated reporter gene. Finally, we demonstrated that the Cβ variants lacking the exon 4 were able to form PKA holoenzymes by associating with the RIIα subunit. However, these PKA holoenzymes did not dissociate, not even in the presence of high non-physiological levels of cAMP.

Abbreviations

AKAP	A kinase anchoring protein
APP	β A4 amyloid protein precursor
ATP	adenosine 5’-triphosphate
C subunit	catalytic subunit
cAMP	3’-5’-cyclic adenosine monophosphate
cDNA	complementary DNA
CNS	central nervous system
CRE	cAMP-regulated element
CREB protein	CRE-binding protein
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides
ECL	enhanced chemiluminescence
EDTA	ethylenedinitro tetraacetic acid
Epac	exchange protein directly activated by cyclic AMP
EST	expressed sequence tags
IC50	inhibitory concentration 50
IP	immunoprecipitation
K_m	Michaelis-Menten constant
LCPUFA	long-chain polyunsaturated fatty acids
L-LTP	late phase of long-term potentiation
luc	luciferase
mRNA	messenger ribonucleic acid
NT2	Ntera 2
NES	nuclear export signal
OD	optical density
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
PAC	plasmid artificial chromosome

PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood leukocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	1-phosphatidylinositol 3-kinase
PKA	protein kinase A
PKI	protein kinase inhibitor
PMSF	phenylmethanesulfonyl fluorid
PrKX	protein kinase X
PVDF	polyvinylidene fluoride
RA	retinoic acid
Rap	Ras-related protein
RNA	ribonucleic acid
rpm	rounds per minute
R subunit	regulatory subunit
RT	reverse transcription
SDS	sodium dodecyl sulphate
TBE	Tris Borate EDTA
UW GCG	University of Wisconsin Genetics Computer Group
WB	Western blot

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1. Introduction

1.1 The cAMP and cAMP-dependent protein kinase signalling pathway

All cells need a tight regulation and coordination of cellular processes. One of the most important mechanisms to achieve this is by protein phosphorylation and dephosphorylation by kinases and phosphatases, respectively (Krebs & Beavo, 1979). A number of kinases and phosphatases have been discovered and characterised. In 1968, a cyclic AMP-dependent protein kinase (PKA) was purified (Walsh *et al.*, 1968). This kinase has been shown to regulate a wide range of cellular and physiological processes, like metabolism, cell proliferation and differentiation, gene regulation and sperm motility (Skålhegg & Taskén, 2000). The PKA-pathway is one of the best studied intracellular signalling pathways in eukaryotic cells and is involved in regulation of cellular functions in nearly all mammalian tissues (Taskén & Aandahl, 2004).

The binding of a ligand to a sevenspan transmembrane G-protein coupled receptor can activate or inhibit the enzyme adenylyl cyclase (AC). AC catalyses the formation of 3'-5'-cylic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) and activation of this enzyme will increase the intracellular cAMP-level. Cyclic AMP can directly activate certain cyclic nucleotide ion channels (Nakamura & Gold, 1987, DiFrancesco & Tortora, 1991) and the guanine nucleotide exchange factors Epac1 and Epac2 which activate the small G-protein Rap1 (Kawasaki *et al.*, 1998). Nevertheless, PKA is considered the main cAMP-target in the cell and cAMP exerts most of its effects through the PKA-pathway. The intracellular concentration and localisation of cAMP is tightly controlled by various phophodiesterases which inactivate cAMP by degrading it to 5'-AMP (Taskén & Aandahl, 2004).

The inactive PKA is a holoenzyme consisting of a regulatory (R) subunit dimer and two catalytic (C) subunits (fig. 1.1). Two major types of PKA exist, PKAI and PKAII, containing different R subunits, RI and RII, respectively. Each of the R subunits can bind two molecules of cAMP in a positive cooperative fashion. Binding of cAMP induces a conformational change in the R-dimer and this releases the C subunits (Corbin *et al.*, 1975). Free C subunits can phosphorylate serine and threonine residues on specific target proteins and thus modulate their function. PKA is a kinase with rather broad specificity, and how distinct cellular effects are achieved has been widely investigated during the last decades (reviewed Taskén & Aandahl, 2004).

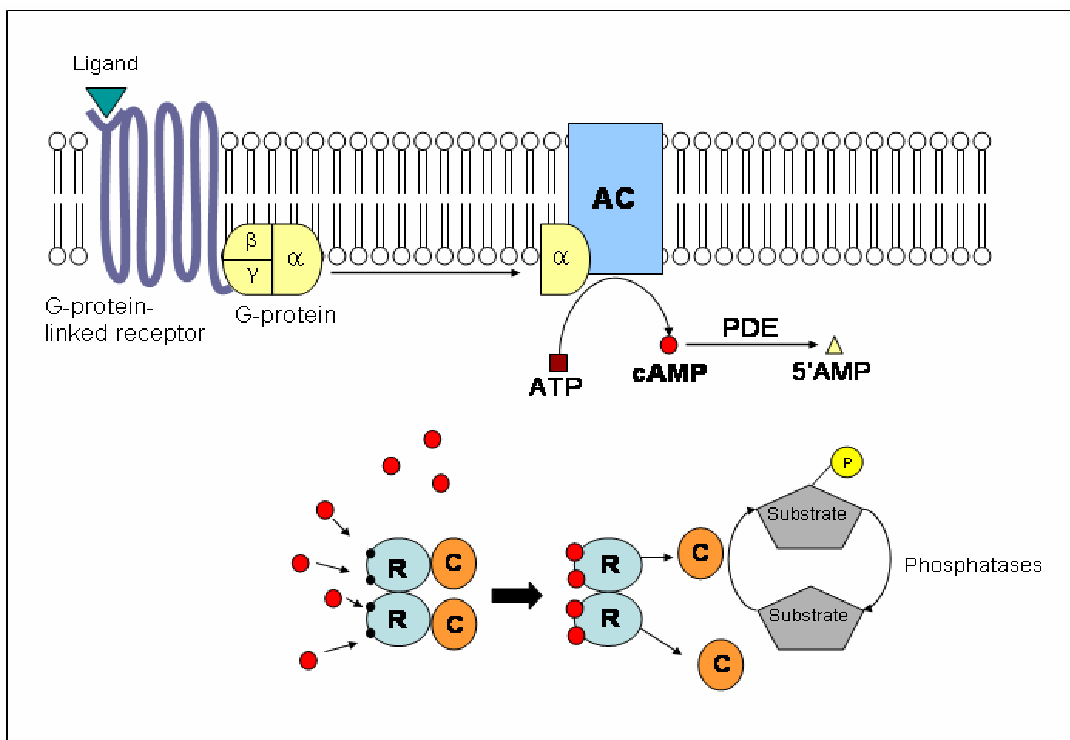

Figure 1.1 The cAMP-PKA pathway

Binding of a ligand to a G-protein-coupled receptor leads to the activation of the enzyme adenylyl cyclase (AC) which catalyses the formation of cAMP from ATP. Cyclic AMP can bind to the regulatory subunit (R) and this releases the catalytic subunit (C). Free C subunits are catalytically active kinases that phosphorylate substrate proteins. The signalling pathway is for example inactivated when cAMP is degraded to 5'AMP by phosphodiesterases (PDE) and when substrate proteins are dephosphorylated by phosphatases.

In the cytosol, free C subunits can be inactivated by reassociation with the R-dimer, by degradation, or they can be transported into the nucleus by passive diffusion and regulate gene transcription (Meinkoth *et al.*, 1990), often by phosphorylating and activating the cAMP-response element binding (CREB) protein. C subunit activity can also be regulated by the endogenous protein kinase inhibitor protein (PKI). PKI binds to the catalytic domain of all C subunits, except for Cγ (Beebe *et al*., 1992). PKI also transports C subunits out of the nucleus as it contains a nuclear export signal (NES) (Wen *et al.*, 1995).

1.2 PKA splice variants

In humans, a total of four genes for the R subunit have been identified; RIα, RIβ, RIIα and RIIβ. Also, four different genes for the C subunit exist; Cα, Cβ, Cγ and X-chromosome encoded protein kinase X (PrKX), which all share the same core catalytic domain. Splice variants of Cα and Cβ have been demonstrated. The Cα gene encodes two known splice variants while the Cβ gene transcribes ten known splice variants. Cα1 and Cβ1 appear to be ubiquitously expressed. On the other hand, Cα-s is only expressed in sperm cells (Reinton *et al.*, 2000). Cβ2 mRNA levels are highest in lymphoid tissues (Ørstavik *et al*., 2001). Neurone-specific Cβ variants are also found; Cβ3, Cβ3b, Cβ3ab, Cβ3abc, Cβ4, Cβ4b, Cβ4ab and Cβ4abc (Kvissel *et al.*, 2004). Cγ expression is restricted to testis (Beebe *et al.*, 1990).

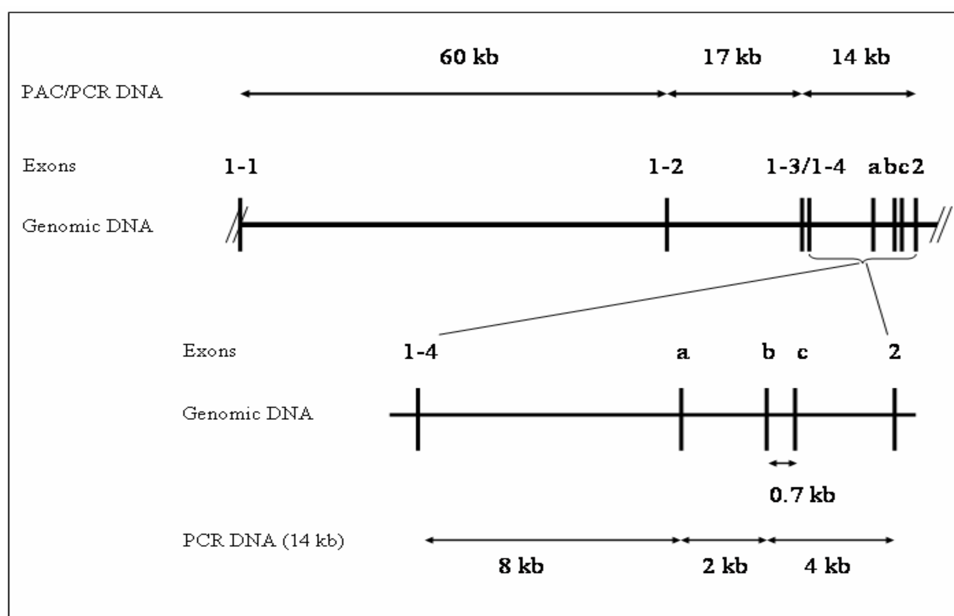

Figure 1.2 The Cβ gene structure upstream of exon 2

The different Cβ exons 1 are drawn as vertical lines and the introns are drawn to scale. Taken from Ørstavik *et al*. (2001), with permission.

The C subunits have different N-terminal domains due to differential splicing of exon 1 (fig. 1.2 and 1.3). This may introduce unique isoform and splice variant specific features like myristoylation, phosphorylation and possible variations in enzyme kinetics. Cα1 and Cβ1 have myristoylation sites in this region and myristoylation is believed to be important for stabilisation of the C subunit and also serves to increase the lipophilic properties. Cα1 and Cβ1 also share a conserved autophosphorylation motif in exon 1, and the isoforms are 91% identical in amino acid sequence (Skålhegg & Taskén, 2000). Nonetheless, Cα1 has a 3-5-fold lower K_m for certain peptide substrates than the Cβ1, in addition to a 3-fold lower IC_{50} for inhibition by PKI and RIIα (Gamm *et al.*, 1996). This demonstrates that there may be unique features associated with the various C subunits even though their amino acid sequences are highly homologous.

Sequence	Variant
MGNAATAKKGSEVES VKEFLAKAK	Cβ1
MAAYR...NDSLHFSEHTALWDRS MKEFLAKAK	Cβ2
MGL VKEFLAKAK	Cβ3
MGL ASCSSSEIS VKEFLAKAK	Cβ3b
MGL SRKSSDASACSSSEIS VKEFLAKAK	Cβ3ab
MGL SRKSSDACACSSSEIS DSF VKEFLAKAK	Cβ3abc
MS VKEFLAKAK	Cβ4
MS ACSSSEIS VKEFLAKAK	Cβ4b
MS ARKSSDASACSSSEIS VKEFLAKAK	Cβ4ab
MS ARKSSDASACSSSEIS DSF VKEFLAKAK	Cβ4abc

Figure 1.3 Amino acid sequence of the N-terminal end of Cβ splice variants
The figure depicts the exon 1 amino acid sequence for Cβ1, Cβ2, Cβ3, Cβ3b, Cβ3ab, Cβ3abc, Cβ4, Cβ4b, Cβ4ab and Cβ4abc and the first amino acids encoded by exon 2. Exon 1 of Cβ2 is shortened. Adapted from Kvissel *et al.* (2004), with permission.

PKA mediates a wide range of physiological effects, some of them are general and others are cell and tissue specific. An obvious question is how specificity is maintained in the PKA-pathway. The existence of a large number of R and C subunits, some of them tissue-specifically expressed, opens for the possibility for many holoenzyme combinations, each of them with different biochemical properties like subtle variations in substrate preferences and phosphorylation kinetics. PKA-targeting to subcellular compartments and substrates via the A-kinase anchoring proteins (AKAPs) clearly contributes to the spatial and temporal specificity. The AKAPs can associate with the R subunits and more than 50 different AKAPs have been identified (Taskén & Aandahl, 2004). Dual-specific AKAPs and AKAPs for either RI or RII have been found, but it is generally believed that RII is the major interacting partner as this has a multifold higher affinity for different AKAPs than RI. The existence of AKAPs is critical, as they also serve as scaffolding proteins and assemble PKA together with other regulatory signalling molecules (Michel & Scott, 2002). Nevertheless, the C subunits are released from both the R subunits and possible AKAPs upon cAMP-binding, and it is likely that other mechanisms necessary to obtain specificity exist.

1.3 Nutrients and the nervous system

Adequate nutrition - sufficient amounts and a right composition of nutrients - is crucial in regulating all physiological processes; growth, development and tissue maintenance. Malnutrition or starvation for a longer period will influence normal cell and tissue functions. This can have several serious consequences during foetal, postnatal and adult life (Modern Nutrition in Health and Disease, Shils *et al*. Lippincott, Williams & Wilkins, 1998.).

The molecular effects of inadequate nutrition are very complex and often not fully understood. Nonetheless, it is known that different amino acids and vitamins act as precursors for hormones and neurotransmitters, or are involved in their biosynthesis. Nutrients can also regulate gene transcription. Obviously, lack of essential nutrients may lead to multiple and serious defects in the development and maintenance of body organs, including the nervous system (Guesry, 1998). In particular, the brain has to undergo several critical periods in its anatomical, biochemical and physiological development. During different stages, the presence and the level of specific nutrients may be of crucial importance, especially during foetal and early postnatal life. In particular, the development of the brain from the last trimester of gestation until the second year of postnatal life is critical in order for an individual to avoid intellectual and/or behavioural impairments (Modern Nutrition in Health and Disease, Shils *et al*. Lippincott, Williams & Wilkins, 1998).

The links between malnutrition and abnormal development of the nervous system are poorly understood. Fortunately, brain development seems fairly resistant to minor nutritional deficiencies. On the other hand, the brain has a particular high metabolic rate and is dependent on continuous metabolism (Basic neurochemistry, Siegel *et al.*, Lippincott, Williams & Wilkins, 1999). Inadequate nutrition can affect both brain metabolism and the building of brain structures. For example, adequate supply of the essential long-chain polyunsaturated fatty acids (LCPUFA), especially docosahexaenoic acid (DHA), is crucial for the building of neural structures and the

retina. Some LCPUFAs are also precursors for eicosanoid production. In addition, fatty acid composition in tissues affect the physical properties of cell membranes and hence membrane-related processes like transport systems, ion channels, receptor function, different signal transduction pathways and enzymatic activity (Wainwright, 2002, Uauy & Castillo, 2003). Iron deficiency, a very prevalent nutritional deficiency, leading to anaemia also affects the nervous system and impairs the psychomotor development in infants and children (Guesry, 1998). Folic acid has been strongly linked to neuronal development, as the frequency of neural tube defects is markedly higher in newborns whose mothers are folate deficient during pregnancy. Folic acid supplementation reduces the incidence of babies born with neural tube defects significantly (Fernstrom, 2000). In addition, different amino acids, small molecules and many micronutrients regulate neuronal activity, by functioning as precursors and cofactors in fundamental biochemical pathways, like the synthesis of neurotransmitters and hormones (Guesry, 1998, Fernstrom, 2000, Zeisel, 2000). For example, tryptophan and choline are precursors for the neurotransmitters serotonin and acetylcholine, respectively.

Several hormones and neurotransmitters exert their effects through activation of a number of membrane receptors, including the G-protein-coupled receptors. Through the cAMP and PKA second messenger pathway, G-protein-coupled receptors regulate essential cellular processes, such as metabolism, cell proliferation and differentiation and gene regulation.

1.4 Vitamin A and the development of the nervous system

The term vitamin A is used for all compounds that have the same biological effects as retinol. Vitamin A is metabolised to other derivatives, such as all-trans retinoic acid, 9-cis retinoic acid, retinaldehyd, 11-cis retinal, anhydroretinol and many more. In the diet, vitamin A is usually provided as carotenoids (from plant sources) and retinylesters (from animal sources) (Mat og Medisin, Bjørneboe & Drevon, Høyskoleforlaget, 1999).

Vitamin A has various functions in humans; as a chromophore in the retina, for normal spermatogenesis, reproduction and embryonic development, for proper haematopoiesis, for gene regulation, cell differentiation and general growth. Already in 1933, vitamin A was identified to be essential for normal embryogenesis (Hale, 1933). Twenty years later it was also found to be teratogenic (Cohlan, 1953).

Retinoic acid (RA), through activation of specific nuclear receptors, is essential for both embryonic and adult growth. Various animal and cell models, in addition to human incidences of vitamin A hypo- or hypervitaminoses, have provided insight to the phleiotropic effects of vitamin A in embryonic development (Zile, 2001, McCaffery *et al.*, 2003). It is obvious that both vitamin A deficiency and excess can be harmful to the developing and adult central nervous system (CNS) (McCaffery *et al.*, 2003). Experiments with various strains of embryonic, neuroblastoma and teratocarcinoma cells have shown that RA induces undifferentiated cells into neurones and glia. RA added to mature neuronal cells can increase the number and length of neurites. Experiments on rat, chicken and quail demonstrate that severe RA deficiency produces a wide range of CNS abnormalities; the caudal hindbrain fails to develop, there is a reduction in neurite outgrowth, the spinal cord is abnormal and the neural crest cells undergo extensive apoptosis. (McCaffery *et al.*, 2003). RA has also been shown to be indispensable for cardiac, vessel, limb, ocular, lung and kidney development (Zile, 2001).

1.5 Multiple roles for PKA in the nervous system

A growing body of evidence supports an important role for PKA in nerve cell development and maintenance. PKA has been shown to be a critical regulator for neuronal and glial differentiation in the developing brain and several neuronal cell lines. Cyclic AMP is one of numerous signalling molecules involved in regulation of nerve cell growth and differentiation, and these effects are at least in part mediated by activation of PKA (De Jonge *et al*., 2001, Kao *et al*., 2002, Sánchez *et al*., 2003, Tojima *et al*., 2003). The mechanisms by which cAMP regulates these events are far from fully understood, but it is likely that cAMP acts by activation of both PKA and Epacs. This will in turn regulate the expression of a number of genes via the CREB protein which binds to cAMP responsive elements (CRE) in the DNA. The CREB family of transcription factors is believed to play an important role in several processes relevant to the function of the nervous system (Lonze & Ginty, 2002). CREB seems to be essential to survival of different neuronal subtypes. This effect may result from a CREB-mediated regulation of pro-survival factors. A model where the activation of CREB shifts the intracellular balance between survival- and death-promoting factors in favour of those that support survival has been suggested. Experiments have shown reduced axonal growth in the absence of CREB. CRE-sequences have been found in the regulatory parts of numerous genes, ranging from genes encoding neurotransmitters, growth factors and hormones, structural proteins and enzymes involved in cellular metabolism (Lonze & Ginty, 2002).

The PKA enzyme itself has also been shown to have a number of effects on neurones. Upregulation of PKA type II and increased PKA activity may participate in the RA-induced growth inhibition and morphological changes seen in SH-SY5Y human neuroblastoma cells (Kim *et al.,* 2000). The same cell line was used in experiments demonstrating that both PKA and 1-phosphatidylinositol 3-kinase (PI3K) play an essential role in cAMP-mediated neurite elongation (Sánchez *et al.,* 2004). Treatment of HiB5 hippocampal progenitor cells with dibutyryl-cyclic AMP and over-expression of Cα, induced neuronal differentiation of the cells, including neurite

outgrowth (Kim *et al.,* 2002). Activation of PKA is also sufficient to induce neuroendocrine-like differentiation of the LNCaP prostate tumor cell line (Bang *et al*., 1994, Cox *et al.,* 2000). Treatment with cAMP-increasing factors resulted in rapid morphological changes in NTera2 (NT2) cells, including the development of axonal processes with a larger diameter (De Jonge *et al.,* 2001). Our laboratory has shown that NT2 cell differentiation to NT2-N nerve cells is followed by the induction of neuron specific Cβ splice variants and increased PKA activity (Kvissel *et al.,* 2004). It may seem like nerve cell differentiation and PKA expression are two processes acting synergistically.

One of the mechanisms behind the cAMP and PKA effects can be the phosphorylation of synapsins. Synapsins are neuronal-specific phosphoproteins that regulate neuronal development and synaptic transmission, and are among the most abundant PKA substrates in adult nerve cells. Three members of the synapsin family have been identified and they are all excellent PKA substrates. They seem to be involved in neuronal development as well, but the level of expression is much lower in developing than in mature neurones. Differentiation of NT2 cells to NT2-N cells increases the expression of synapsins (Leypoldt *et al.,* 2002). Experiments with *Xenopus laevis*-embryos, demonstrate that the nerve growth-promoting effect of cAMP and PKA is partly mediated by phosphorylation of synapsins at a single amino acid residue (Kao *et al.,* 2002).

Recently it was shown that the Cβ splice variant Cβ4ab interacts with the neurotrophin receptor $p75^{NTR}$ (Higuchi *et al.,* 2003). Neurotrophins are nerve growth factors that regulate cell differentiation, growth, survival and apoptosis. Cβ4ab-phosphorylation of $p75^{NTR}$ on serine 304 promotes translocation of the receptor to lipid rafts, an element essential to the effects of neurotrophins.

Finally, PKA activity in the hippocampus and the amygdala is believed to play an important role in learning and memory. $C\beta1^{-/-}$ mice show impaired hippocampal plasticity (Qi *et al.,* 1996). Another mouse model has shown that normal PKA expression in the hippocampus is important for the late phase of long-term potentiation (L-LTP) (Abel *et al.,* 1997) and consequently for long-term memory.

2. Aims

How specificity is maintained in the cAMP-PKA pathway is not completely understood. Four genes of the catalytic subunit have been identified in humans, and in NT2-N cells several neuronal-specific Cβ splice variants are expressed. The PKA C subunits which have been comprehensively characterised show different enzyme properties despite highly homologous amino acid sequences. It is therefore likely that tissue-specific expression of different C variants contributes to the precision observed in the cAMP-PKA effects. Cloning experiments led us to believe that even more Cβ variants were expressed in RA-differentiated NT2-N cells. The major aims for this thesis are consequently to:

1) Identify PKA Cβ variants lacking the exon 4 in NT2-N cells differentiated by RA.

2) Develop a method for identifying the expression of novel Cβ splice variants in different tissues.

3) Study the basic characteristics of the novel splice variants, such as catalytic activity and R subunit interaction.

3. Materials and methods

All chemicals were supplied in analytical grade from Sigma-Aldrich unless otherwise stated.

All PCR-reactions were performed on Gene Amp PCR System 9700 from Applied Biosystems.

MilliQ water refers to distilled, ion-exchanged and filtered sterile water.

3.1 Ntera 2 cell culture

Reagents:

Dulbecco's Modified Eagle Medium (Sigma-Aldrich, cat.no. D6545)

Foetal Bovine Serum (Sigma-Aldrich, cat.no. F7524)

Penicillin-Streptomycin Solution 50 x (Sigma-Aldrich, cat.no. P0906)

L-glutamine 200 mM (Sigma-Aldrich, cat.no. G7513)

Protocol:

The human teratocarcinoma cell line Ntera 2 (NT2 cl.D1, ATCC cat.no. CRL-1973) was cultured in an incubator at 37ºC in humidified air with 5% CO_2. The growth medium (complete DMEM) consisted of Dulbecco's Modified Eagles Medium supplemented with 10% Foetal Bovine Serum (heat-inactivated at 56ºC for 30 minutes), 2 mM L-glutamine and Penicillin-Streptomycin, 50 U/ml and 50 µg/ml, respectively. The cells were subcultured by trypsination twice a week. Neuronal NT2 cells, NT2-N cells, were received from Dr. Terje Rootwelt, Department of Pediatric Research, Rikshospitalet. The cells were differentiated by RA as described by Kvissel *et al*. (2004).

3.2 293T cell culture

Reagents:

RPMI 1640 (Sigma-Aldrich, cat.no. R0883)

Foetal Bovine Serum (Sigma-Aldrich, cat.no. F7524)

Penicillin-Streptomycin Solution 50 x (Sigma-Aldrich, cat.no. P0906)

L-glutamine 200 mM (Sigma-Aldrich, cat.no. G7513)

Non-essential amino acids solution 10 mM (GibcoBRL, cat.no. 11140-035)

Sodium pyruvate 100 mM (GibcoBRL, cat.no. 11360-039)

Protocol:

The highly transfectable human epithelial cell line 293T (ATCC, cat.no. CRL-11268), was cultured in an incubator at 37ºC in humidified air with 5% CO_2. The growth medium (complete RPMI 1640 medium) consisted of RPMI 1640 supplemented with 10% Foetal Bovine Serum (heat-inactivated at 56ºC for 30 minutes), 2 mM L-glutamine, 0.1 mM Non-essential amino acids, 1 mM Sodium Pyruvate and Penicillin-Streptomycin, 50 U/ml and 50 μg/ml, respectively. The 293T cells were usually split 1:5 three times a week by mechanical dislodgment.

3.3 Isolation of total RNA

Reagents:

RNeasy Mini Kit (Qiagen, cat.no. 74106)

β-mercaptoethanol

Protocol:

The procedure described in the RNeasy Mini Kit was followed using pelleted 5 x 10^6 NT2-N cells. 600 µl Buffer RTL containing 1% β-mercaptoethanol was added to each pellet. The cells were homogenised by passing the lysate 10 times through a 23 G needle fitted to a syringe. 1 x volume (600 µl) 70% ethanol was added and the sample was applied to an RNeasy mini column, which was centrifuged. The flow-through was discarded and the membrane washed with Buffer RW1 and Buffer RPE. RNA was eluted in 30 µl RNase-free water. The amount of RNA isolated was quantified by UV absorbance at 260/280 nm (Ultrospec 3100 pro, Amersham Biosciences).

3.4 Reverse Transcription

Reagents:

Reverse Transcriptase System (Promega, cat.no. A3500).

Protocol:

Complementary DNA synthesis was performed as described by the manufacturer. $MgCl_2$ solution, Reverse Transcription 10x buffer, dNTP Mixture, Recombinant Rnasin Ribonucelase Inhibitor, AMV Reverse Transcriptase, Random Primers, 1 µg RNA and Nucelase-Free water were mixed in amounts as described. The RNA was pre-incubated at 70ºC for 10 minutes. The Reverse Transcriptase mixture was incubated at room temperature for 10 minutes and then at 42ºC for 15 minutes. The sample was heated at 95ºC for 5 minutes and then incubated on ice for another 5 minutes to inactivate the enzyme.

3.5 Polymerase Chain Reaction (PCR)

Reagents:

10 x buffer (Finnzymes, cat.no. F-511)

Deoxyribonucleotide (dNTP) mix 25 mM (Finnzymes, cat.no. F-560XL)

DyNAzyme II DNA Polymerase 2 U/µl (Finnzymes, cat.no. F-501L)

Primers (10 µM):

Upper primer Cα (binds Cα specific sequence in exon 3:
5’CGGGAACCACTATGCC3’, Sigma-Genosys Ltd, non-commercial)

Lower primer Cα (binds Cα specific sequence in exon 6:
5’GTAGCCCTGCTGGTCAATGA3’, Sigma-Genosys Ltd, non-commercial)

U1: Upper primer Cβ1 (binds Cβ1 specific sequence in exon 1:
5’CCCTTCTTGCCATCG3’, Sigma-Genosys Ltd, non-commercial)

U2: Upper primer Cβ2 (binds Cβ2 specific sequence in exon 1:
5’GCCGGTTATTTCATAGACAC3’, Sigma-Genosys Ltd, non-commercial)

U3: Upper primer Cβ3 (binds Cβ3 specific sequence in exon 1:
5’AAGACGTTTAGGTGCAAT3’, Sigma-Genosys Ltd, non-commercial)

U4: Upper primer Cβ4 (binds Cβ4 specific sequence in exon 1:
5’CCCTTTGCTGTTGGAT3’, Sigma-Genosys Ltd, non-commercial)

U5: Upper primer Cβ common (binds Cβ specific sequence in exon 3:
5’ACACAAAGCCACTGAA3’, Sigma-Genosys Ltd, non-commercial)

L1: Lower primer Cβ2 (binds Cβ specific sequence in exon 8:
5’CCTAATGCCCACCAATCCA3’, Sigma-Genosys Ltd, non-commercial)

L2: Lower primer Cβ common (binds Cβ specific sequence in exon 9: 5'TTCCGTAGAAGGTCCTTGAG3', Sigma-Genosys Ltd, non-commercial)

Protocol:

The PCR reaction mixtures contained 2.5 µl cDNA sample, 2.5 µl 10 x buffer, 1 mM dNTP mix, 1 U DyNAzyme II DNA Polymerase, 0.4 µM upper primer, 0.4 µM lower primer and MilliQ water to a final volume of 25 µl. All PCR reactions were run using the following cycling conditions: initial denaturation: 95ºC for 2 minutes, amplification: 95ºC for 30 seconds, 60ºC for 30 seconds and 72ºC for 2 minutes (30 cycles) and final elongation at 72ºC for 10 minutes.

3.6 Agarose gel electrophoresis

Reagents:

BDH Electron Agarose (VWR International Ltd, cat.no. 44366 5W)

Ethidium bromide (2.5 mg/ml)

123 bp DNA ladder (Invitrogen, cat.no. 15613-011)

1 Kb DNA ladder (Invitrogen, cat.no. 15615-016)

High DNA Mass ladder (Invitrogen, cat.no. 10496-016)

10 x TBE buffer	6 x Orange DNA loading dye
0.45 M Tris-Borate	20% Ficoll 400 (VWR International Ltd, cat.no. 437092S)
0.1 M EDTA	0.25% Orange G
	20 mM EDTA

Protocol:

The BioRad submerged horizontal agarose gel system was used. 1% agarose gels were made by mixing 1 g of agarose powder in 100 ml of 1 x TBE. The mixture was heated to 100 ºC and was boiled for two or three minutes until a clear solution was obtained. The liquid gel was left to cool approximately 15 minutes. 10 µl ethidium bromide (2.5 mg/ml) was added and the mixture was poured into a previously assembled gel caster and left to set for about 30 minutes. The ready agarose gel was placed in an electrophoresis tray filled with 1 x TBE. DNA-samples were mixed with 6 x Orange DNA loading dye and loaded into the wells of the agarose gel. The gels were subjected to electrophoresis at 90 V for 30-40 minutes. The DNA-bands were visualised using a UV-transluminator (Saveen).

3.7 Screening for short Cβ variants

Reagents:

10 x buffer (Finnzymes, cat.no. F-511)

Deoxyribonucleotide (dNTP) mix, 25 mM (Finnzymes, cat.no. F-560XL)

DyNAzyme II DNA Polymerase (2 U/µl) (Finnzymes, cat.no. F-501L)

Restriction enzyme SspI (Invitrogen, cat.no. 15457-011), including 10 x restriction buffer REACT 6

Total RNA from human peripheral leukocytes (kindly provided by Tilahun Tolesa Hafte)

Total RNA from NT2-N cells (from 3.3 Isolation of total RNA)

Human Brain Poly A^+ RNA (BD Biosciences Clontech, cat.no. 6516-1)

Primers (10 µM):

U5: Upper primer Cβ common (see 3.5 Polymerase Chain Reaction (PCR))

L2: Lower primer Cβ common (see 3.5 Polymerase Chain Reaction (PCR))

Protocol:

The screening method is an assay created especially for this work and combines PCR and restriction enzyme cutting. The intention is to enhance the amplification of Cβ PCR fragments lacking the exon 4.

Complementary DNA was synthesised from RNA as described in 3.4 Reverse Transcription. The first PCR reaction was mixed and performed with the same cycling conditions as described in 3.5 Polymerase Chain Reaction (PCR), but with 20 cycles. 5 µl of the PCR reaction was mixed with 2 µl 10 x buffer REACT 6, 2 µl SspI restriction enzyme and MilliQ water up to 20 µl. The mixture was incubated at 37ºC overnight. Then, 2.5 µl of the cutting reaction was used as template to set up a new PCR reaction otherwise similar to the first, this time with 35 cycles. The resulting DNA fragments were run on an agarose gel. If restriction digestion was insufficient, as judged by the intensity of the upper band, the template was redigested for another night before the second PCR reaction was set up again.

3.8 Generation of expression vectors

3.8.1 Amplification of Cβ3ab and cloning into entry vector pENTR D-TOPO

Reagents:

PfuUltra High Fidelity polymerase (2.5 U/µl) with PfuUltra 10 x buffer (Stratagene, cat.no. 600380)

Deoxyribonucleotide (dNTP) mix, 25 mM (Finnzymes, cat.no. F-560XL)

pENTR Directional TOPO Cloning Kits with One Shot TOP10 Chemically Compentent *E.coli* (Invitrogen, cat.no. 2400-20)

BrsGI (New England Biolabs, cat.no. R0575) with NE buffer 2

Primers (10 µM, PAGE-purified):

Upper primer Cβ3ab (binds Cβ3ab in exon 1: 5'CACCGCCGCCACCATGGGATTGTCACGCAAATCATCAGATGCATCT3', Sigma-Genosys Ltd, non-commercial)

Lower primer Cβ3ab 1 (binds Cβ3ab in exon 10 and includes stop codon: 5'TTAAAATTCACCAAATTCTTTTGCAC3', Sigma-Genosys Ltd, non-commercial)

Lower primer Cβ3ab 2 (binds Cβ3ab in exon 10, does not include stop codon; 5'AAATTCACCAAATTCTTTTGCACATT3', Sigma-Genosys Ltd, non-commercial)

LB-medium and LB-agar (500 ml):

2.5 g Yeast Extract Granulated (VWR International Ltd, cat.no. 536204K)

5 g Peptone from casein pancreatically digested (VWR International Ltd, cat.no. 536005F)

5 g Sodium Chloride (VWR International Ltd, cat.no. 102415K)

1.5 ml 1 M NaOH

500 ml distilled water

For LB-agar: 7.5 g agar-agar (VWR International Ltd, cat.no. 1.101614)

The solution is autoclaved and cooled. The antibiotic (Kanamycin (50 µg/µl) (GibcoBRL, cat.no. 11815-024)) was added to a final concentration of 50 µg/ml.

Protocol:

Initially, two different PCR-reactions were performed using the same upper primer, but different lower primers. The PCR reaction mixtures contained 1 µl cDNA from NT2-N cells, 5 µl 10 x Pfu buffer, 0.2 µM of each primer, 0.5 mM dNTP mix, 2.5 U Pfu Ultra High Fidelity Polymerase and MilliQ water to a final volume of 50 µl. The PCR reactions were run on the following cycling conditions: initial denaturation: 95ºC for 1 minute, amplification: 95ºC for 30 seconds, 50ºC for 30 seconds and 72ºC for 3 minutes (30 cycles) and final elongation at 72ºC for 7 minutes. The reaction using lower primer Cβ3ab 1 yielded no visible PCR-products. Nevertheless, both reactions were cloned into pENTR D-TOPO vector in accordance with the manufacturer’s protocol: 4 µl of the PCR reaction product was mixed with 1 µl salt solution and 1 µl TOPO vector. The reaction was incubated 5 minutes at room temperature and then placed on ice. Transformation of One Shot TOP10 cells was done according to the chemical transformation protocol: 2 µl of the cloning reaction was added to 1 unit of One Shot TOP10 Chemically Competent Cells and incubated on ice for 15 minutes. The bacteria were heat-shocked at 42ºC for 30 seconds and then immediately placed on ice. 250 µl of SOC medium was added and the samples incubated horizontally at 37ºC in a shaker at 250 rpm for 1 hour. The cells were then seeded on LB-agar with Kanamycin and incubated overnight at 37ºC. We only obtained colonies after cloning with the PCR-products from the reaction using lower primer Cβ3ab 2 without stop codon. Bacterial colonies created from one single transformed cell were selected and grown at 37ºC for 6-24 hours in a miniculture consisting of 3 ml LB-medium with Kanamycin. DNA was isolated by MiniPrep as described in a following section and subjected to control restriction cutting with BsrGI following the manufactorer’s standard procedure and run on an agarose gel. As we wanted to express Cβ3ab without a tag, MiniPrep DNA from properly transformed *E.coli* was therefore used in another restriction cutting reaction followed by ligation, to create a Cβ3ab insert with stop codon. One clone was sent to Medigenomix GmbH for control sequencing before the insert was transferred into an expression vector by the LR-Clonase reaction, see 3.10.

3.8.2 The ExSite Method

Reagents:

ExSite PCR-Based Site-Directed Mutagenesis Kit (Stratagene, cat.no. 200502) with XL-1-Blue supercompetent *E.coli*.

Cβ1 in pENTR D-TOPO vector (control sequenced and tested plasmid number 102 from plasmid collection, provided by Sigurd Ørstavik)

HA-tagged Cα1 and Cβ1 in pEF BOS-HA (kindly provided by Vibeke Sundvoll, Institute of Immunology, Rikshospitalet)

EcoRI (Invitrogen, cat.no. 15202-013) with buffer H

BrsGI (New England Biolabs, cat.no. R0575) with NE buffer 2

LB-medium and LB-agar (see 3.8.1 Amplification of Cβ3ab and cloning into entry vector pENTR D-TOPO)

Kanamycin (50 µg/µl) (GibcoBRL, cat.no. 11815-024)

Ampicillin (50 µg/µl) (Sigma-Aldrich, cat.no. SA 2804)

<u>Mutagenesis primers (10 µM, all primers are 5’ phosphorylated and PAGE-purified):</u>

Upper primer Cα (binds Cα specific sequence in exon 5: 5’GACAACTCAAACTTATACAT3’, Sigma-Genosys Ltd, non-commercial)

Lower primer Cα (binds Cα specific sequence in exon 3: 5’CTTCTGTTTGTCGAGGATCTT3’, Sigma-Genosys Ltd, non-commercial)

Upper primer Cβ (binds Cβ specific sequence in exon 5: 5’GATAATTCTAATTTATACATGGT3’, Sigma-Genosys Ltd, non-commercial)

Lower primer Cβ (binds Cβ specific sequence in exon 3: 5’CTTCTGCTTATCTAAGATCTTCA3’, Sigma-Genosys Ltd, non-commercial)

Protocol:

Use of the ExSite method took advantage of our already created expression vectors. In the ExSite method, specially designed mutagenesis primers can be used to introduce point mutations, create small or large deletions or to make insertions into double stranded DNA (fig. 3.1).

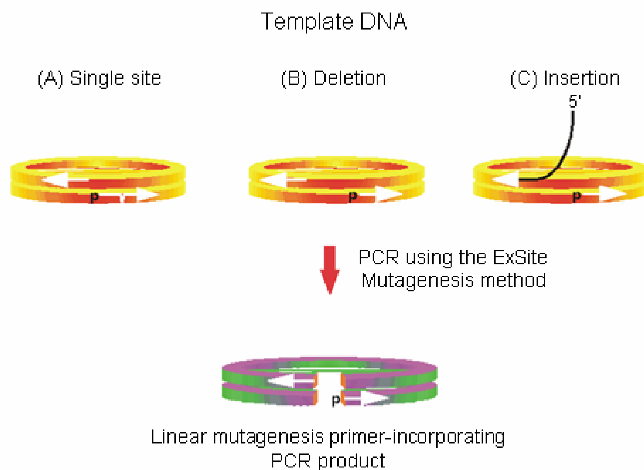

Figure 3.1 Different uses of the ExSite method
Depending on primer design, the ExSite method can be used to make 3 different modifications of existing vectors. We used it to make a large deletion. Taken from the Stratagene ExSite protocol with permission.

Primers precisely flanking the Cα and Cβ exon 4 sequences were designed. The ExSite protocol was followed. Plasmid DNA was used in a PCR-mixture containing mutagenesis buffer, dNTP mix, oligonucleotide primers and ExSite DNA polymerase blend. Mutagenesis cycling parameters were chosen as recommended by the manufacturer. After completion of the PCR, two populations of plasmids existed; the desired linear plasmids with the primers incorporated, and the parental plasmids which had not incorporated the mutagenesis primers (fig. 3.2). DpnI restriction enzyme and cloned Pfu DNA polymerase were added to the reaction at the same time, and the reaction was first incubated for 30 minutes at 37ºC and then for 30 minutes at 72ºC. The DpnI enzyme, which has methylated adenine bases (5'-G^{m6}ATC-3') as target sites, digested the parental plasmid population as DNA from almost all *E.coli* strains, including from the *E.coli* strains we use, is *dam* methylated. PCR-generated linear plasmids with mutagenesis primers incorporated remained undigested. The cloned Pfu DNA polymerase end-polished the remaining DNA, that is extended bases placed on the 3' ends of the PCR products by the ExSite DNA polymerase blend were removed. Mutagenesis buffer, rATP and MilliQ water

were added to the reaction and mixed thoroughly. 10 µl of the reaction mixture was then transferred to a fresh microcentrifuge tube, added T4 DNA ligase and finally incubated for one hour at 37ºC. This created circularised, double stranded plasmids with the targeted alteration.

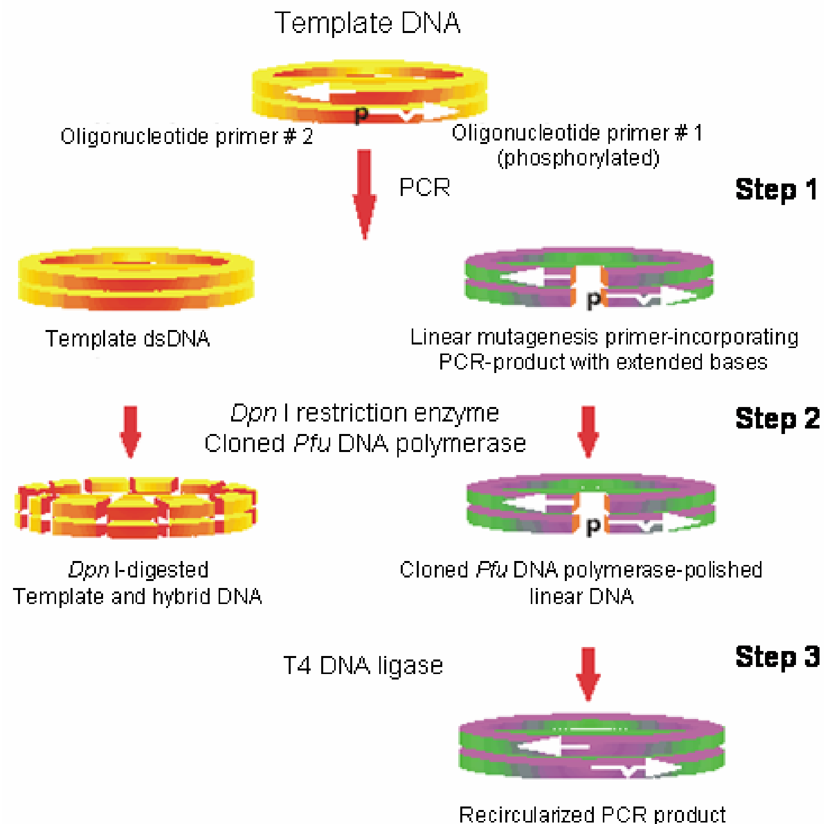

Figure 3.2 Schematic representation of the different steps in the ExSite protocol
Schematic drawing of the sequential steps in the ExSite method. From the Stratagene ExSite protocol with permission.

The ligated DNA was then transformed into XL-1-Blue supercompetent cells according to the protocol: 2 µl ligase-treated DNA was mixed with 80 µl competent cells and incubated on ice for 30 minutes. The bacteria were heat-shocked at 42ºC for 45 seconds and immediately placed on ice. The cells were diluted 1:10 in SOC-medium and incubated horizontally at 37ºC in a shaker at 250 rpm for 1 hour before being plated on LB-agar plates with the proper antibiotic (Kanamycin for the pENTR D-TOPO clones and Ampicillin for the pEF BOS-HA clones) and incubated overnight. The mutagenised colonies were then grown overnight in minicultures and MiniPrep DNA was prepared (see 3.11.1). The DNA was controlled by restriction

enzyme cutting (BsrGI for the pENTR D-TOPO vectors and EcoRI for the pEF BOS-HA vectors) according to the manufactorer's standard procedure and run on an agarose gel before selected clones were shipped to Medigenomix GmbH for sequencing.

3.9 TA-cloning and sequencing

Reagents:

TA Cloning Kit with the TA cloning vector pCR2.1 (Invitrogen cat.no. K2040-01), including One Shot Top10 Chemically Competent *E.coli* and SOC medium.

LB-medium and LB-agar (see 3.8.1 Amplification of Cβ3ab and cloning into entry vector pENTR D-TOPO)

Kanamycin (50 μg/μl) (GibcoBRL, cat.no. 11815-024)

Ampicillin (50 μg/μl) (Sigma-Aldrich, cat.no. SA 2804)

Protocol:

The manufacturer's protocol was followed: TA ligation reactions were prepared with the amounts of PCR product, salt solution, sterile water and cloning vector as described. The mixture was incubated for 15 minutes at room temperature. Transformation of One Shot Top 10 *E.coli* was done according to the chemical transformation protocol described under 3.8.1 Amplification and cloning into entry vector pENTR D-TOPO. Bacterial colonies created from one single transformed cell were picked and grown overnight in microtiter plates. PCR reactions using the Cβ common primer pair and agarose gel electrophoresis were performed to identify the insert. Selected clones were shipped to Medigenomix GmbH for nucleotide sequencing.

3.10 LR Clonase Reaction

Reagents:

LR Clonase Enzyme Mix (Invitrogen, cat.no. 11791), including LR Clonase Enzyme Mix, Proteinase K solution, 5 x LR Clonase Reaction Buffer and pENTR-gus Positive Control plasmid

Destination vector pEF-DEST51 (Invitrogen, cat.no. 12285-011)

Library Efficiency DH5α Competent Cells (Invitrogen, cat.no. 18263-012)

MiniPrep DNA of pENTR D-TOPO vectors with different inserts; Cβ3abΔ4 and Cβ1Δ4 (created in 3.8.1 and 3.8.2, respectively)

LB-medium and LB-agar (see 3.8.1 Amplification of Cβ3ab and cloning into entry vector pENTR D-TOPO)

Ampicillin (50 µg/µl) (Sigma-Aldrich, cat.no. SA 2804)

BrsGI (New England Biolabs, cat.no. R0575) with NE buffer 2

Protocol:

Our reactions contained 4 µl 5 x LR reaction buffer, 300 ng destination vector (pEF-DEST51), 4 µl LR Clonase Enzyme Mix, 8 µl MilliQ water and 2 µl MiniPrep DNA or 100 ng pENTR-gus as a positive control. The reaction mixture was incubated at 25ºC for 1 hour. To stop the reaction, 2 µl Proteinase K solution was added to each sample followed by incubation at 37º for 10 minutes. 1 µl of the reaction was then used in a transformation reaction with 50 µl Library Efficiency DH5α Competent Cells. 30 minutes incubation on ice was followed by heat-shock for 30 seconds at 42ºC. 450 µl SOC medium was added to the cells and the samples incubated horizontally at 37ºC in a shaker at 250 rpm for 1 hour before being plated on LB-agar plates with ampicillin and incubated at 37ºC overnight. A few bacterial

colonies were picked and grown in a miniculture for 6-24 hours and DNA isolated by MiniPrep (see 3.11.1). The DNA was controlled by restriction cutting by BsrGI in accordance with the manufactorer's standard protocol and run on an agarose gel.

3.11 Plasmid DNA purification

3.11.1 MiniPrep

Reagents:

Jetquick Plasmid MiniPrep Spin Kit (Genomed, cat.no. 400250)

LB-medium (see 3.8.1 Amplification of Cβ3ab and cloning into entry vector pENTR D-TOPO)

Kanamycin (50 µg/µl) (GibcoBRL, cat.no. 11815-024)

Ampicillin (50 µg/µl) (Sigma-Aldrich, cat.no. SA 2804)

Protocol:

Bacterial cells were grown in minicultures of 3 ml LB-medium with proper antibiotic (50 µg/ml) overnight. Cells were harvested by centrifugation at 16.000 x g for 3 minutes and the medium was removed. The MiniPrep protocol was then followed: the cells were resuspended in G1 solution, lysed in G2 solution and G3 was added for neutralisation. The mixture was centrifuged at 16.000 x g for 10 minutes and the supernatant was loaded to the spin column and centrifuged at 16.000 x g for 1 minute. The column was washed once with G4 solution before plasmids were eluted with 50 µl MilliQ water preheated to 70ºC.

3.11.2 MaxiPrep

Reagents:

EndoFree Plasmid Maxi Kit (Qiagen, cat.no. 12362)

LB-medium (see 3.8.1 Amplification of Cβ3ab and cloning into entry vector pENTR D-TOPO)

Kanamycin (50 µg/µl) (GibcoBRL, cat.no. 11815-024)

Ampicillin (50 µg/µl) (Sigma-Aldrich, cat.no. SA 2804)

Protocol:

Large scale DNA preparations were performed using the EndoFree Plasmid Maxi Kit as described by the manufacturer. In brief: bacterial cultures were grown overnight in 250 ml LB-medium with selective antibiotics (50 µg/ml) and with vigourous shaking (250 rpm) at 37ºC. The bacterial cells were harvested by centrifugation at 5000 x g for 25 minutes and the LB-medium was removed. All the subsequent steps were done according to the protocol. Resuspension of the pellet was followed by lysis and neutralisation. The lysate was filtrated and then treated with endotoxin removal buffer. The endotoxin treated, filtered lysate was then applied to an equilibrated QIAGEN-tip 500 column and allowed to empty by gravity flow. The plasmids attach to the column, which was washed twice before the DNA was eluted. Isopropanol was added to precipitate the DNA and the mixture was centrifuged at 15000 x g for 30 minutes at 4ºC. The pellet was then washed twice in 1.5 ml endotoxin-free 70% ethanol and air-dried before it was allowed to resuspend in MilliQ water at 4ºC overnight. DNA concentration was determined by UV spectrophometry (Ultrospec 3100 pro, Amersham Biosciences) and quantitative analysis on an agarose gel.

3.12 Transfection of 293T cells by Lipofectamine 2000

Reagents:

Optimem medium (GibcoBRL, cat.no. 31985-047)

Lipofectamine 2000 (Invitrogen, cat.no.11668-019)

RPMI 1640 (Sigma-Aldrich, cat.no. R0883)

Foetal Bovine Serum (Sigma-Aldrich, cat.no.F7524)

L-glutamine 200 mM (Sigma-Aldrich, cat.no. G7513)

Non-essential amino acids solution 10 mM (GibcoBRL, cat.no. 11140-035)

Sodium pyruvate 100 mM (GibcoBRL, cat.no. 11360-039)

Protocol:

The transfection medium (RPMI 1640 medium for transfection) consisted of RPMI 1640 supplemented with 10% Foetal Bovine Serum (heat-inactivated at 56ºC for 30 minutes), 2 mM L-glutamine, 0.1 mM Non-essential amino acids and 1 mM Sodium Pyruvate. The transfections were done according to Lipofectamine 2000 protocol, using 6-well cell culture plates with a surface area of 10 cm^2 per well. One day before transfection, 0.7 x 10^6 293T cells were plated in 1.5 ml transfection medium so that they would be 90-95% confluent at the time of transfection. For each well, 4 µg DNA and 10 µl Lipofectamine 2000 was diluted separately in 250 µl Optimem, gently mixed and incubated at room temperature for 5 minutes. The diluted DNA and the diluted Lipofectamine 2000 were combined and mixed gently, then incubated for 20 minutes in room temperature. 500 µl of the DNA-Lipofectamine 2000 mix was added to each well containing cells and transfection medium to a final volume of 2 ml per well and mixed carefully. The cells were incubated 20-24 hours in humidified air with 5% CO_2 at 37ºC and then harvested.

3.13 Preparation of cell lysates

Reagents:

Phosphate buffered saline (PBS):

(Phosphate buffered saline tablets, P-4417, Sigma-Aldrich, Inc. dissolved in distilled water. Contains 0.01 M phosphate buffer, 0.0027 KCl, 0.137 M NaCl, pH 7.4)

2 x /3 x SDS loading buffer:

100 mM/150 mM Tris

0.4%/0.6% bromphenol blue

10%/15% β-mercaptoethanol

20%/30% glycerol

4%/6% SDS (sodium dodecyl sulphate)

Buffer A:

150 mM NaCl

50 mM Tris, pH 7.4

Protease inhibitor cocktail (Sigma-Aldrich, cat. no. SP8340)

0.5% Triton X-100

Buffer B:

100 mM NaCl

50 mM Tris, pH 7.4

5 mM EDTA

50 mM NaF

50 mM NaPP

1 mM Na_3VO_4

1 mM PMSF

0.5% Triton X-100

Protease inhibitor cocktail (Sigma-Aldrich, cat. no. SP8340)

Protocol:

10 x 10^6 cells were harvested for preparation of lysates for immunoprecipitation and 5 x 10^6 cells were harvested for preparation of lysates for kinase assays. After aspiration of the transfection medium, the cells were harvested by scraping in 1 ml PBS and then centrifuged at 800 x g for 5 minutes. PBS was removed and the cells were washed two more times in 1 ml PBS. The cell pellets were resuspended in 400 µl buffer A (for immunoprecipitations) and 250 µl buffer B (for kinase assays). The cells were lysed by thoroughly vortexing and incubation on ice for 30 minutes. The lysate was centrifuged at 16.000 x g for 30 minutes to remove cell debris. All centrifugation steps were done at 4ºC. 30 µl of the lysates for kinase assay was added 30 µl 2 x SDS loading buffer, boiled at 96ºC for 5 minutes and subjected to Western blotting (see 3.15).

3.14 Determination of protein concentration

Reagents:

Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, cat.no. 500-0006)

Protein Standard II (Bio-Rad, cat.no. 500-0007)

Protocol:

The assay was performed diluting 6 concentrations of bovine serum albumin -1, 2, 4, 6, 8 and 10 µg protein - in 800 µl distilled H_2O to create a standard curve. The cell lysates from kinase assays were diluted to suitable concentrations depending on the expected protein amount, typically 1:10. 10 µl diluted lysate was mixed with 790 µl distilled water. 200 µl of the Bio-Rad reagent was added, the samples vortexed and incubated for 5 minutes. Optical density (OD) at wavelength 595 nm was measured with Ultrospec 3100 pro from Amersham Biosciences using disposable plastic cuvettes. All measurements were done in duplicates.

3.15 SDS-PAGE and immunoblotting (Western blotting)

Reagents:

SuperSignal West Pico Chemiluminescent (Pierce, prod.no. 34080)

SuperSignal West Dura Extended Duration Substrate (Pierce, prod.no. 34076)

BenchMark Prestained Protein Ladder (Invitrogen, cat.no.10748010)

Immobilion polyvinylidene fluoride (PVDF) membrane (Millipore, cat.no. IPVH00010)

Hyperfilm MP (Amersham Biosciences, cat.no. RPN1675K)

Primary antibodies:

Anti-Cmono (tdl), mouse monoclonal IgG (Transduction Laboratories, cat.no. 610981), dilution 1:500

Anti-HA, mouse monoclonal antibody (Covance/Berkely Antibody Company BAbCO, cat.no.MMS101R), dilution 1:5000

Secondary antibody:

Peroxidase-conjugated goat affinity-purified antibody to mouse IgG (ICN Biomedicals, cat.no. 55563), dilution 1:2000

SDS running buffer:	Transfer buffer:	Washing buffer:
25 mM Tris-HCl	39 mM Tris-base	10 mM Tris-base
250 mM glycine	48 mM glycine	150 mM NaCl
0.1% SDS	20% methanol	0.1% Tween 20
		pH 7.5

Blocking buffer:

5% Nestlé Molico Instant non-fat dry milk powder in Washing buffer

Protocol:

12.5% polyacrylamid (BioRad, Criterion Precast Gels) gels were used, with 18 or 12 wells. 25 µl (18 wells gel) or 30 µl (12 wells gel) of prepared samples were loaded to gels and subjected to electrophoresis in SDS running buffer at 130 V for approximately 2 hours. The separated proteins were transferred to PVDF membranes by electro blotting at 100 V for 1 hour in Transfer buffer. The membranes were incubated in Blocking buffer for 1 hour at room temperature or overnight at 4ºC. Primary antibody was diluted in Washing buffer and the membranes incubated for 1 hour in room temperature. Excess antibodies were removed by washing the blots in Washing buffer for 6 x 10 minutes before the membranes were incubated for 1 hour at room temperature with secondary antibody diluted in Washing buffer. The blots were then washed in Washing buffer for another 6 x 10 minutes. Immunoreactive proteins were visualised using the ECL detection system and mulitpurpose Hyperfilm MP. The films were developed with the Kodak X-omat 1000 Processor.

3.16 Immunoprecipitation

Reagents:

Immunoprecipitations (IP) were performed in buffer A (see 3.13 Preparation of whole cell lysates)

3 x SDS loading buffer (see 3.13 Preparation of whole cell lysates)

Anti-RIIα (poly), rabbit polyclonal IgG (Skålhegg *et al.,* 1992), dilution 1:100

Rabbit IgG, unspecific rabbit polyclonal IgG (Sigma-Aldrich, I-5006), 0.5 µg per sample

Dynabeads Protein G (Dynal Biotech, prod.no. 100.03), dilution 1:10

Protocol:

Cell lysates were prepared as described in 3.13. Preparation of whole cell lysates. Pre-clearing was done to remove proteins with unspecific binding to the Dynabeads; the magnetic Protein G beads were added to the lysate, rotated at 4ºC for 30 minutes and the tubes put into a magnetic holder for approximately 1 minute. Dynabead pellets were removed. The IP was initiated when the antibody was added to the remaining supernatants and the samples were incubated with rotation at 4ºC overnight. Precipitation was completed by incubating the samples with Dynabeads on a rotator for 1 hour at 4ºC and followed by placing the tubes into the magnetic holder. Dynabead pellets were washed 3 times with 300 µl of buffer A. The pellets were then added 30 µl buffer A with or without 1 mM cAMP, incubated 5 minutes before being placed into the magnetic holder and the supernatants were collected. This was repeated once. The pellets were finally added 60 µl of buffer A without cAMP. All pellets and supernatants were then added 30 µl 3 x SDS loading buffer, boiled at 96ºC for 5 minutes and subjected to Western blotting.

3.17 Kinase assay

Reagents:

Kemptide (Sigma-Aldrich, cat.no. K1127)

Histone (Sigma-Aldrich, cat.no. H5505)

γ-[^{32}P]ATP (Amersham Biosciences, cat.no. PB10281)

Cyclic AMP (Sigma-Aldrich, cat.no.A6885)

P81 phosphocellulose paper (Whatman, cat.no. 3698-915)

75 mM phosphorus acid

96% ethanol

Opti-fluor (Packard BioScience, cat.no. 47-040101)

Kinase assay stock solution:

48 ml 50 mM Tris pH 7.4

1.2 ml 1M $MgAc_2$

1.2 ml 10 mM ATP

Protocol:

Kemptide and histone assay mixtures were made from 1029 µl kinase assay stock solution, 2.6 µl γ-ATP (5000 Ci/mmol) and 21 µl of 6.5 mM stock of substrate (kemptide or histone), resulting in a final substrate concentration of 130 µM. The phosphotransferase reaction was started with the addition of 10 µl cell lysate (prepared as described in 3.13 Preparation of whole cell lysates) to 40 µl kinase assay mixture with added cAMP (62.5 µM). The cell lysate samples were adjusted to equal protein concentration. The reaction was incubated for 9 minutes at 30ºC, then 35 µl of the reaction mixture was spotted on a strip of P81 paper and tossed into 500 ml 75 mM phosphoric acid to terminate the reaction. The strips were washed 4 x 10 minutes in 500 ml 75 mM phosphoric acid, once in 200 ml 96% ethanol and air dried. Activity was measured by liquid scintillation in 3 ml Opti-fluor (1900 TR, Packard).

3.18 Luciferase reporter assay

Reagents:

CRE-luc-plasmid (kindly provided by Professor Kristin Austlid Taskén, Aker Urological University Clinic, Aker hospital)

β-galactosidase plasmid (kindly provided by Professor Kristin Austlid Taskén, Aker Urological University Clinic, Aker hospital)

Phosphate buffered saline (PBS) (see 3.13 Preparation of cell lysates)

Reporter Lysis 5 x buffer (Promega, cat.no.E3971)

β-galactosidase (Sigma-Aldrich, cat.no. G1875)

Luciferase assay mix:

470 µM Luciferin (SynChem OHG, cat.no. s039)

0.1 mM EDTA

3.74 mM $MgSO_4$

20 mM Tricine

33.3 mM DTT

530 µM ATP (Boehringer, cat.no. 519979)

270 µM Coenzyme A (Boehringer, cat.no. 103411)

pH 7.8

Z buffer:

60 mM Na_2PO_4

40 mM NaH_2PO_4

10 mM KCl

1 mM Mg_2SO_4

50 mM β-mercaptoethanol

pH 7.0

<u>ONPG buffer:</u>

4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG) in 100 mM Phosphate buffer, pH 7.0

Protocol:

293T cells were transfected with 4 µg DNA; ⅓ CRE-luc plasmid, ⅓ β-gal plasmid and ⅓ Cα1-HA, Cα1-HAΔ4, Cβ1-HA, Cβ1-HAΔ4, Cβ1, Cβ1Δ4, Cβ3ab or Cβ3abΔ4 plasmid, as described in 3.12 Transfection of 293T cells by Lipofectamine 2000. 2.5 x 10^6 transfected cells were harvested, washed 3 times in 1 ml PBS and lysed in 200 µl 1 x reporter lysis buffer by vortexing each sample twice for 15 to 30 seconds. Cell debris was pelleted by centrifugation at maximum speed for 3 minutes. 10 µl of the supernatant and 100 µl of the luciferase assay mix were mixed in a luminometer tube. The amount of emitted light during 10 seconds was measured using a luminometer (TD20/20, Turner Designs).

In order to adjust luciferase activity in relation to the transfection efficiency, the β-galactosidase level in each sample was estimated and compared with a β-galactosidase standard curve. 3 µl β-galactosidase was diluted in 750 µl Z-buffer and this solution was used to create the standard curve. Increasing amounts of β-galactosidase standard solution (0, 10, 20, 30, 40, 50 and 60 µl) was mixed with decreasing amounts of Z-buffer (110, 100, 90, 80, 70, 60, and 50 µl) and 40 µl ONPG buffer in duplicates in a microtiter plate. 10 µl cell lysate was mixed with 100 µl Z-buffer and 40 µl ONPG buffer, also in duplicates. The samples were incubated at 37ºC for 30 minutes followed by measurement of the OD at 405 nm (Titertek Multiscan PLUS).

4. Results

4.1 Identification of novel splice variants of the C subunit

During RA-dependent differentiation of NT2 cells to NT2-N cells, a number of C splice variants were identified, cloned and sequenced (Kvissel *et al.*, 2004). In an attempt to amplify Cβ3ab for expression, a variant of Cβ3ab lacking the exon 4 was identified (Sigurd Ørstavik, personal communication). A comparison of this sequence to a library of sequenced cDNAs (the EST-database, Adams *et al.*, 1991) revealed that a similar clone had previously been reported (accession number AK091420). The fact that these novel cDNAs lacked the exact nucleotide sequence of the exon 4 and had been demonstrated by two independent experiments, led us to believe that these cDNAs reflected mRNAs expressed in human cells and were not an artefact. We therefore decided to confirm the existence of Cβ splice variants lacking the exon 4 and to study the resulting proteins.

4.1.1 Identification of novel C mRNA lacking exon 4 expressed in NT2-N cells

In order to identify the different Cβ splice variants during RA-dependent NT2 cell differentiation, Kvissel *et al.* (2004) used the PCR primers U1, U2, U3 and U4, which correspond to DNA sequences in exon 1-1, 1-2, 1-3 and 1-4, respectively (fig. 4.1), in combination with lower primers in exon 2. As we were searching for splice variants lacking the exon 4, the same upper primers were used, but in combination with lower primers that correspond to sequences in exon 8 or 9 (fig. 4.1, L1 and L2). If a significant amount of any of the Cβ splice variants tested lacked the exon 4, the PCR reaction would yield smaller sized products. These could be identified on an agarose gel due to a higher migration speed. However, one concern had to be considered; the exon cluster abc is alternatively spliced in with exon 1-3 and 1-4 (fig. 1.2), and using the primers U3 and U4 in combination with an exon 9 lower primer would produce a

mixture of DNA products with and without exon 4. This would make it impossible to distinguish the differences according to size determination from agarose gels unless the mixed PCR products were subcloned and sequenced. We therefore also generated and tested a primer pair only recognising a Cβ common sequence; U5 and L2, which corresponds to sequences in exon 3 and exon 9 (fig. 4.1), which would simply detect the presence of exon 4-lacking Cβ splice variants. Furthermore, to test if excluding exon 4 is a Cβ specific phenomenon that does not occur for Cα transcripts, Cα primers were designed; upper primer binding in exon 3 and lower primer in exon 6.

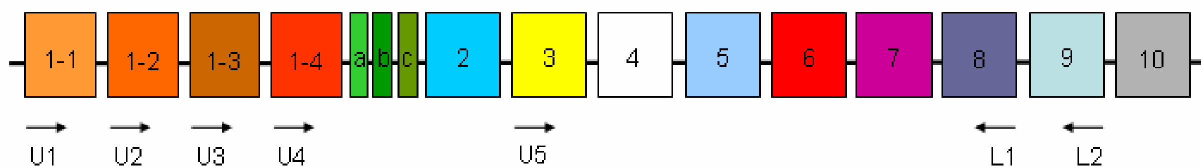

Figure 4.1 The exons of the Cβ gene

Schematic representation of the Cβ exons and the position of the upper primers (U1 to U5) and the lower primers (L1 and L2) used to amplify the different Cβ splice variants.

To further conclude that we would be able to distinguish between PCR products with and without the exon 4 sequence, we calculated the theoretical sizes of the expected PCR products (tab. 4.1). The exon 4 is 99 bases long both in the Cα and the Cβ gene. This implies that we would most probably be able to distinguish relative size differences on an agarose gel. Table 4.1 shows the calculated lengths (base pairs, bp) of the PCR fragments from Cα, Cβ1, Cβ2, Cβ3 and Cβ4 PCR products with and without the exon 4 sequence. Because the exons 1-3 and 1-4 may splice in with the exons a (24 bp), b (24 bp) and c (9 bp) to form the Cβ3 and Cβ4 abc splice variants, this would result in a number of different PCR products, probably visualised as a smear on an agarose gel. The estimated lengths of these PCR products were not included in the table.

Primer pair used	Expected fragment size with exon 4	Expected fragment size without exon 4
Cα primer pair	343 bp	244 bp
Cβ1: U1-L2	838 bp	739 bp
Cβ2: U2-L1	738 bp	639 bp
Cβ3: U3-L2	831 bp	732 bp
Cβ4: U4-L2	834 bp	735 bp
Cβ common: U5-L2	630 bp	531 bp

Table 4.1 The calculated lengths of the amplification fragments
Table showing the theoretical lengths of the respective Cα and Cβ splice variant fragments, with and without the exon 4 sequence, using the different combinations of the Cα primers and the primers U1 to U5 and L1 and L2.

Isolation of total RNA from NT2-N cells was followed by cDNA synthesis and PCR amplification using the primers described in figure 4.1. The results from figure 4.2 demonstrate that PCR products with the lengths listed in table 4.1 were indeed identified for Cα (lane 3) and for all Cβ variants (lanes 4, 5, 6 and 7). Interestingly, all the PCR reactions using the Cβ primers yielded two detectable bands, indicating the presence of alternatively spliced Cβ variants. The PCR reaction using the Cα specific primers produced only one visible band. Together this implies that the exon 4 exclusion does take place for the Cβ variants, but not for the Cα isoforms. It should be noted that the Cβ common primer pair also yielded a double band (fig. 4.2, lane 2), further supporting the hypothesis of the existence of alternatively spliced Cβ variants that lack the nucleotides transcribed from the Cβ exon 4 sequence.

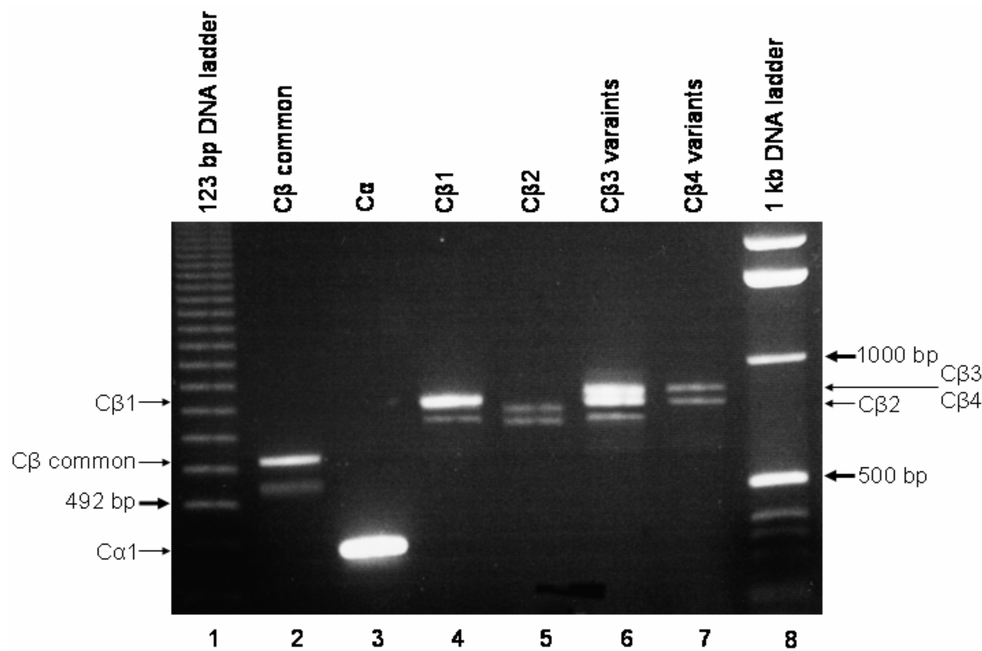

Figure 4.2 PCR amplification demonstrating that alternative splicing occurs for Cβ, but not for Cα

Complementary DNA was generated from total RNA from NT2-N cells and PCR was performed using the primer combinations U1 to U5 and L1 and L2 to amplify the Cβ splice variants and the primer combination exon 3 (upper) and exon 6 (lower) to amplify Cα. The PCR amplified products were separated by agarose gel electrophoresis and visualised by ethidium bromide staining. Thick arrows indicate the migration of the DNA standards and thin arrows indicate the amplification product identity. The lengths of the products are given in table 4.1. Corresponding negative control reactions in which cDNA was not added, were also performed. They did not result in any detectable PCR fragments (not shown).

To validate that the lower Cβ bands did in fact represent a Cβ splice variant, PCR products were cloned into the pCR2.1 vector and sequenced. The sequences were compared with the published Cβ sequences using the University of Wisconsin Genetics Computer Group (UW GCG) software and revealed that several of the Cβ splice variants are expressed without the exon 4 sequence. As an example, figure 4.3 shows the comparison of the amino acid sequences of Cβ3ab to Cβ3ab lacking the exon 4.

```
  1 MGLSRKSSDASACSSSEISVKEFLAKAKEDFLKKWENPTQNNAGLEDFER  50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
  1 MGLSRKSSDASACSSSEISVKEFLAKAKEDFLKKWENPTQNNAGLEDFER  50

 51 KKTLGTGSFGRVMLVKHKATEQYYAMKILDKQ..................  82
    ||||||||||||||||||||||||||||||||
 51 KKTLGTGSFGRVMLVKHKATEQYYAMKILDKQKVVKLKQIEHTLNEKRIL 100

 83 ...............KDNSNLYMVMEYVPGGEMFSHLRRIGRFSEPHARF 117
                   |||||||||||||||||||||||||||||||||||
101 QAVNFPFLVRLEYAFKDNSNLYMVMEYVPGGEMFSHLRRIGRFSEPHARF 150

118 YAAQIVLTFEYLHSLDLIYRDLKPENLLIDHQGYIQVTDFGFAKRVKGRT 167
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 YAAQIVLTFEYLHSLDLIYRDLKPENLLIDHQGYIQVTDFGFAKRVKGRT 200

168 WTLCGTPEYLAPEIILSKGYNKAVDWWALGVLIYEMAAGYPPFFADQPIQ 217
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 WTLCGTPEYLAPEIILSKGYNKAVDWWALGVLIYEMAAGYPPFFADQPIQ 250

218 IYEKIVSGKVRFPSHFSSDLKDLLRNLLQVDLTKRFGNLKNGVSDIKTHK 267
    ||||||||||||||||||||||||||||||||||||||||||||||||||
251 IYEKIVSGKVRFPSHFSSDLKDLLRNLLQVDLTKRFGNLKNGVSDIKTHK 300

268 WFATTDWIAIYQRKVEAPFIPKFRGSGDTSNFDDYEEEDIRVSITEKCAK 317
    ||||||||||||||||||||||||||||||||||||||||||||||||||
301 WFATTDWIAIYQRKVEAPFIPKFRGSGDTSNFDDYEEEDIRVSITEKCAK 350

318 EFGEF 322
    |||||
351 EFGEF 355
```

Figure 4.3 Comparing the deduced amino acid sequences of Cβ3ab with and without the exon 4 sequence Based on results obtained by PCR amplification and subsequent sequencing, a cDNA sequence encoding a Cβ3ab isoform lacking the exon 4 was composed, translated into the amino acid sequence (top) and aligned with the deduced amino acid sequence of Cβ3ab with exon 4 (bottom). The comparison demonstrates that the 33 amino acids corresponding to the exon 4 encoded sequence (red letters) are lacking.

The newly identified Cβ splice variants were named the Δ4 variants of their respective isoform. A total of six novel splice variants were found and were designated Cβ1Δ4, Cβ2Δ4, Cβ3Δ4, Cβ3abΔ4, Cβ3abcΔ4 and Cβ4abΔ4 (fig. 4.4.)

- Cβ1Δ4: 1-1 | 2 | 3 | 5 | 6 | 7 | 8 | 9 | 10
- Cβ2Δ4: 1-2 | 2 | 3 | 5 | 6 | 7 | 8 | 9 | 10
- Cβ3Δ4: 1-3 | 2 | 3 | 5 | 6 | 7 | 8 | 9 | 10
- Cβ3abΔ4: 1-3 | a | b | 2 | 3 | 5 | 6 | 7 | 8 | 9 | 10
- Cβ3abcΔ4: 1-3 | a | b | c | 2 | 3 | 5 | 6 | 7 | 8 | 9 | 10
- Cβ4abΔ4: 1-4 | a | b | 2 | 3 | 5 | 6 | 7 | 8 | 9 | 10

Figure 4.4 Schematic overview of the confirmed CβΔ4 variants Schematic representation of the Cβ splice variants Cβ1, Cβ2, Cβ3, Cβ3ab, Cβ3abc and Cβ4ab lacking the exon 4 sequence that are expressed in NT2-N cells differentiated by RA. The different exons are indicated with different colours and numbers/letters.

4.1.2 Identification of CβΔ4 variants in human brain

NT2 cells differentiate into neurones by RA and all known Cβ splice variants, except Cβ2, are expressed at high levels in cells of nervous tissue. Furthermore, immune cells, such as human peripheral blood leukocytes (PBL), express Cβ1 and Cβ2 mRNA (Ørstavik *et al*., 2001) and we identified Cβ1Δ4 and Cβ2Δ4 in the differentiated NT2-N cells. Together this implied that human brain cells and PBL might express CβΔ4 variants and we investigated whether the CβΔ4 variants were present in these neuronal and non-neuronal cell types.

Total RNA from PBL and Poly A^+ RNA from human brain were used in cDNA synthesis followed by PCR amplification. NT2-N cells were included as a control. Only the Cβ common primer combination U5 and L2 was applied as we only wanted to investigate whether any CβΔ4 variants were present or not, and it was not important to distinguish between them. The results demonstrated that NT2-N and human brain cells, but not human PBL, express a shorter Cβ fragment (fig. 4.5, lanes 2, 3 and 4). These PCR products most likely represent CβΔ4 variants, and imply that human brain, but not PBL, express Cβ splice variants lacking the exon 4 sequence. This indicates that exclusion of the exon 4 may be a phenomenon specific to nerve cells.

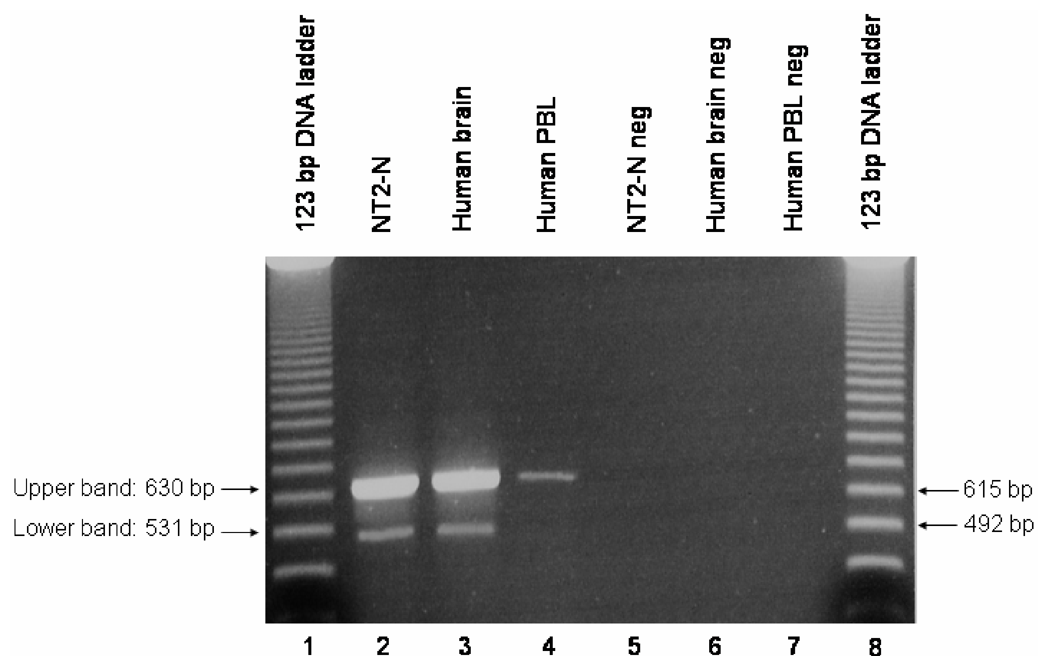

Figure 4.5 Human brain cells express Cβ splice variants lacking the exon 4 sequence
Complementary DNA was made from NT2-N cells, human brain cells and human PBL RNA. PCR amplification was performed using the Cβ common primers. The PCR products were run on an agarose gel and this demonstrated that the Cβ common primers generated two products of 630 and 531 base pairs for NT2-N and human brain cells (lanes 2 and 3), but not for human PBL (lane 4). According to expected sizes, they represent Cβ and CβΔ4, respectively. To the negative controls, cDNA was not added (lanes 5, 6 and 7). Arrows on the right indicate migration of the DNA standard and arrows on the left indicate migration of the amplification products.

4.1.3 Development of a sensitive method for screening for CβΔ4 variants in human tissues

Amplification of cDNA from RNA demonstrated that human brain cells and NT2-N cells express Cβ lacking the exon 4. However, the amplification of the long PCR fragment (containing the exon 4) might obscure the presence of low levels of Cβ mRNA lacking exon 4. We therefore decided to develop a more sensitive method to detect the presence of CβΔ4 variants. In the initial step, Cβ variants are amplified by PCR using the Cβ common primer pair U5 with L2. This would result in a DNA fragment population mostly consisting of normal Cβ variants. To increase the possibility of detecting any CβΔ4 variants, we took advantage of the fact that the restriction enzyme SspI has a unique cutting site in the Cβ exon 4 sequence. By

applying this enzyme to the amplified Cβ DNA, all variants containing the exon 4 would be cleaved, whereas the fragments lacking the exon 4 would remain intact. After SspI treatment, the remaining CβΔ4 variants, when undergoing a second round of PCR, would be selectively amplified to a detectable level to establish whether they are in fact expressed in the tissue tested (fig. 4.6).

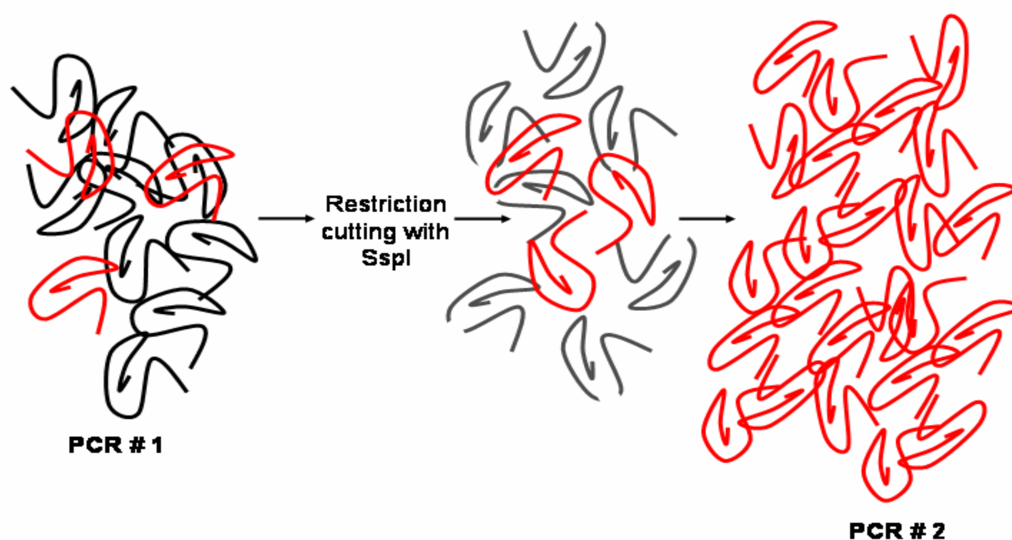

Figure 4.6 Schematic representation of selective amplification of CβΔ4 splice variants to increase detection sensitivity

An initial PCR reaction will yield a DNA population mainly consisting of Cβ fragments containing the exon 4 (black) and a minor population of CβΔ4 fragments (red) if they are expressed. Restriction enzyme cutting with SspI in exon 4 will cut Cβ forms containing the exon 4 in two, making it impossible to amplify these variants in a second PCR round. The second PCR reaction, using the SspI treated DNA as template and Cβ common primers, will amplify the remaining CβΔ4 variants.

In the screening assay experiments we used cDNA synthesised from RNA from NT2-N cells, human brain cells and PBL. Negative control reactions for both the initial PCR reaction and the restriction cutting were also performed, yielding four different sample combinations for each cell type (fig. 4.7); the first without cDNA and not treated with enzyme, the second without cDNA and treated with enzyme, the third with cDNA and not treated with enzyme and the fourth with cDNA and treated with enzyme. As expected, the samples with no cDNA resulted in blank lanes (fig. 4.7, lanes 2, 3, 6, 7, 10 and 11). When cDNA from the different cell types was included in the first PCR reaction, but without treatment with the SspI enzyme, this

resulted in the upper Cβ common band (fig. 4.7, lanes 4, 8 and 12). However, when the PCR reactions were treated with the SspI prior to the second PCR round, this yielded a lower Cβ common band for NT2-N cells and human brain cells, whereas for PBL, no band was observed (fig. 4.7, lanes 5, 9 and 12). The latter results strengthen the conclusion from figure 4.5, that CβΔ4 variants exist in differentiated NT2-N cells and human brain cells, but is absent in PBL. It should be noted from the figure that a weak upper Cβ common band is still present in the sample for the human brain cells (fig. 4.7, lane 9), indicating that the digestion of the normal Cβ fragments was not complete.

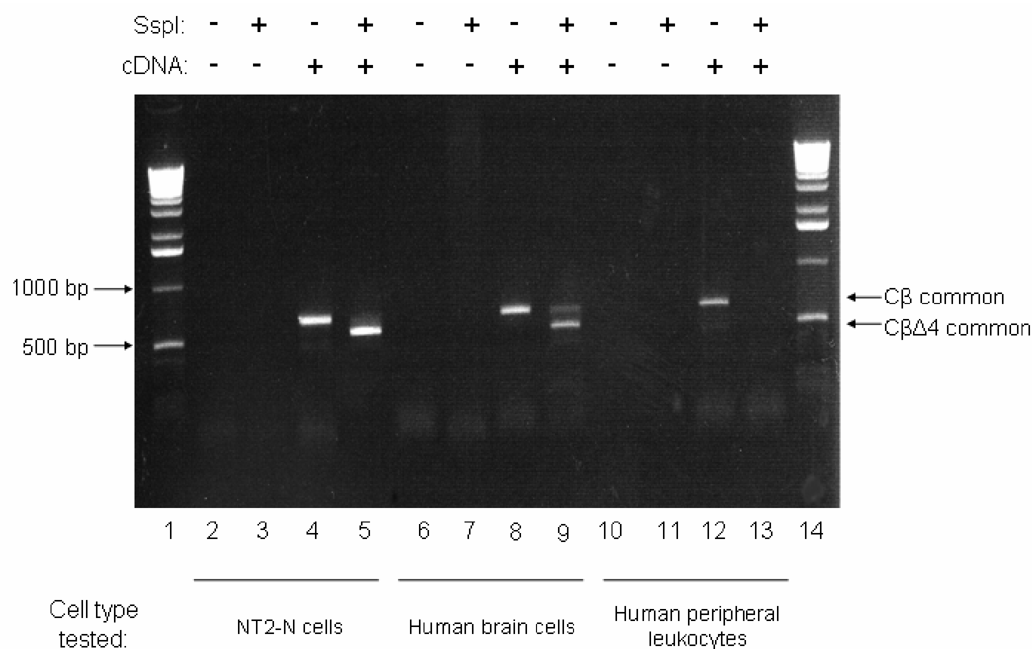

Figure 4.7 CβΔ4 variants are expressed in nerve cell tissue but not in peripheral blood leukocytes

Complementary DNA was synthesised from RNA from NT2-N cells, human brain cells and human peripheral leukocytes. PCR amplifications were performed using the Cβ common primers. DNA was either treated with SspI (+) or left untreated (-), re-amplified with the Cβ common primers and the DNA products were run on an agarose gel. In re-amplified reactions not treated with SspI, a 630 bp DNA fragment was detected for all cell types tested (lanes 4, 8 and 12). In re-amplified reactions treated with SspI, a 531 bp DNA fragment was identified for NT2-N cells and human brain cells (lanes 5 and 9), but not for PBL (lane 13). It should be noted that a weak 630 bp band was also detected in lane 9. This is indicative of an incomplete digestion of exon 4 containing fragments in this reaction. Arrows on the left indicate migration of the DNA standard and arrows on the right indicate amplification product identity.

4.2 Functional characterisation of the CΔ4 subunit kinase activity

As the presence of the CβΔ4 variants was established, we questioned what might be their biological significance. We studied the three dimensional (3D) structure for Cα (Knighton *et al.*, 1991) using the Cn3D version 4.1 software (National Center for Biotechnology Information). We found that the exon 4 sequence encodes an α-helix which defines the outer border of the catalytic domain (fig. 4.8, yellow line).

Consequently, we expected that the catalytic cleft of the CβΔ4 variants would have a significantly altered organisation. This would most probably affect the function of the protein and may result in a radically changed catalytic activity.

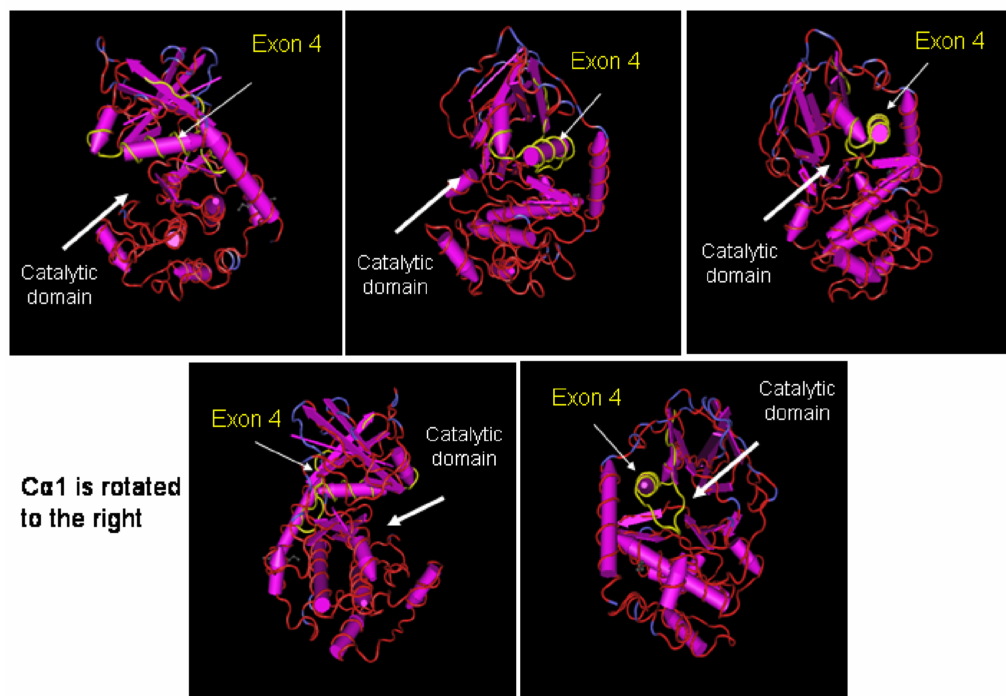

Figure 4.8 Three dimensional structure of Cα1
The 3D structure of Cα 1 rotated 360°. The exon 4 encoded sequence is outlined in yellow and is indicated by a thin arrow. Thick arrows indicate the catalytic cleft.

In order to explore the functional features of different CΔ4 variants, the respective Δ4 variants of HA-tagged Cα1 and Cβ1 in pEF BOS-HA expression vector were generated. Untagged Cβ1 and Cβ3ab and their Δ4 variants were generated in the pENTR D-TOPO vector of the Gateway system (Invitrogen), recombined into the pEF DEST51 eukaryotic expression vector and were also expressed in eukaryotic cells. Despite the fact that CαΔ4 variants were not detected by our previous experiments, we decided to make Cα-HAΔ4 for the functional experiments.

The main known functions of the catalytic subunits of PKA are their ability to transfer the γ-phosphate group from ATP to serine and threonine residues on specific proteins and peptides and their ability to form complexes with the R subunits. We decided to investigate these two key characteristics.

4.2.1 In vitro activity – kinase assay

Cell lysates made from 293T cells overexpressing Cα1-HA, Cα1-HAΔ4, Cβ1-HA and Cβ1-HAΔ4 were monitored for PKA-specific phosphorylation activity in the presence of the synthetic peptide kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) which contains a conserved PKA phosphorylation site (Kemp *et al.,* 1977). This demonstrated that despite being correctly expressed (fig. 4.9, panel A), neither Cα1-HAΔ4 nor Cβ1-HAΔ4 were kinetically active against kemptide (fig. 4.9, panel B). This was also the case when the same proteins were tested against histone (fig. 4.9, panel C) which is a protein containing several naturally occurring PKA phosphorylation sites (Zhang *et al*., 2004). We noted that Cα1-HA shows a substrate preference to kemptide, being virtually inactive against histone. Together this suggests that deletion of the exon 4 results in inactive C subunits. However, these experiments were all done with N-terminally HA-tagged C subunits and the tag may have influenced catalytic activity. We have experienced that tagging the C subunits may affect the kinase activity in various degrees. Based on this, non-tagged Cβ1, Cβ1Δ4, Cβ3ab and Cβ3abΔ4 were also overexpressed in 293T cells (fig. 4.10, panel A). As Cβ3ab is a recently identified Cβ splice variant, its enzyme activity had never been tested before. Preliminary experiments were performed and established that Cβ3ab was in fact an active kinase as determined by the kinase assay and the luciferase reporter assay (results not shown). Then, the Cβ1, Cβ1Δ4, Cβ3ab and Cβ3abΔ4 enzyme activities were collectively monitored against kemptide and histone (fig. 4.10, panel B and C), revealing once more that the Δ exon 4 splice variants totally lack enzyme activity above the endogenous C subunit activity reflected in the mock transfected cells. Taken together, these results strongly suggest that deletion of the exon 4 sequence results in catalytically inactive PKA C subunits.

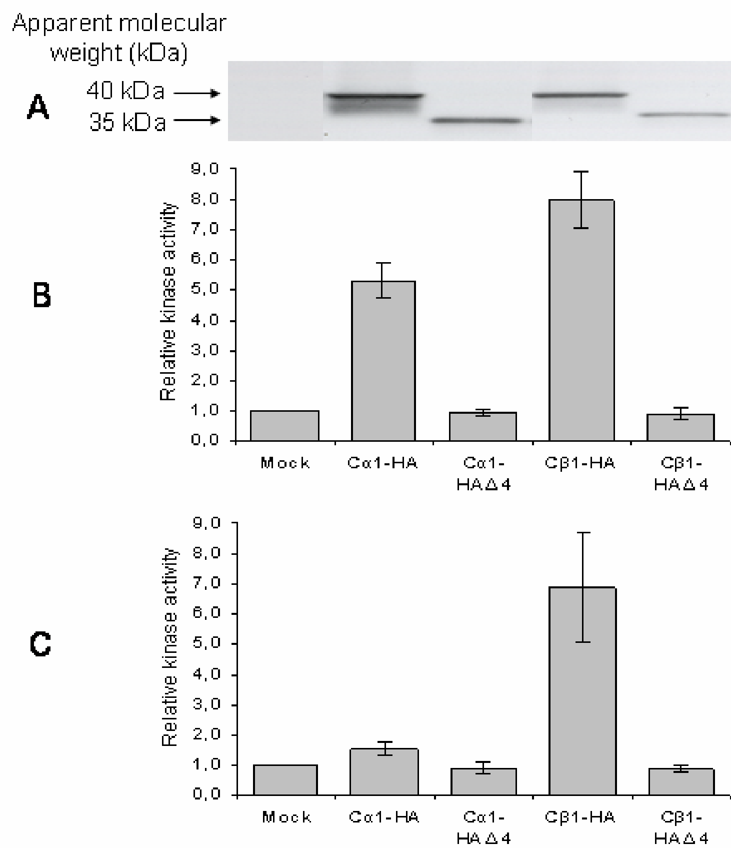

Figure 4.9 Expression and catalytic activities of HA-tagged Cα1 and Cβ1 with and without the exon 4 sequence

A) 293T cells were transfected with vectors expressing Cα1-HA, Cα1-HAΔ4, Cβ1-HA and Cβ1-HAΔ4. Cell lysates were analysed for HA-immunoreactive proteins by SDS-PAGE (12.5% gels) and immunoblotting using anti-HA. This revealed that all variants were expressed at detectable levels and with the expected relative sizes (kDa) as indicated by arrows. B) Cell extracts as shown in A) were monitored for PKA-specific kinase activity against the synthetic PKA-substrate kemptide and against histone (C). Relative kinase activities were measured in relation to total PKA activity in mock transfected cells and are presented as the average from three representative experiments, +/- the standard error of the mean (SEM).

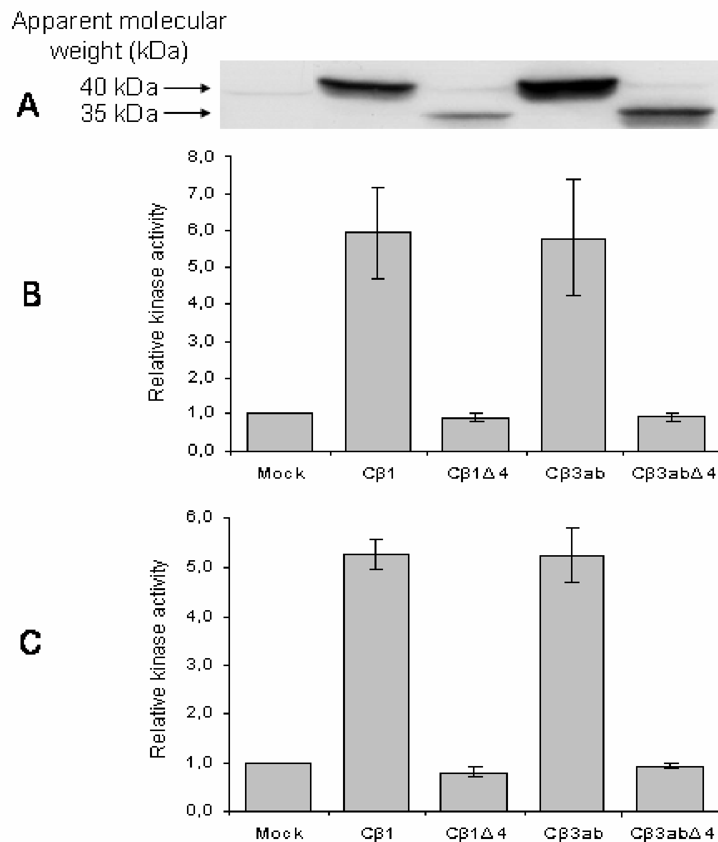

Figure 4.10 Expression and catalytic activities of native Cβ1 and Cβ3ab with and without the exon 4 sequence
A) 293T cells were transfected with vectors expressing native Cβ1, Cβ1Δ4, Cβ3ab and Cβ3abΔ4. Cell lysates were analysed for C subunit immunoreactive proteins by SDS-PAGE (12.5% gels) and immunoblotting using anti-Cmono. This revealed that all variants were expressed at detectable levels and with the expected relative sizes (kDa) as indicated by arrows.
B) Cell extracts as shown in A) were monitored for PKA-specific kinase activity against the synthetic PKA-substrate kemptide and against histone (C). Relative kinase activities were measured in relation to total PKA activity in mock transfected cells and are presented as the average from three representative experiments, +/- the standard error of the mean (SEM).

4.2.2 Induction of a CRE-regulated promotor

Our kinase assay experiments clearly indicated that the CΔ4 variants are completely catalytically inactive against kemptide and histone. Because such assays are pure *in vitro* analyses and may lack factors important for the CΔ4 variants to function, we decided to test their ability to regulate the endogenous activity of a CRE-regulated promoter cloned in front of a luciferase reporter gene. In this assay, cells are transfected with a CRE-luc reporter plasmid where the expression of the luciferase enzyme is under the control of a CRE-element. Co-transfection with the HA-tagged and native C subunits and their respective Δ4 variants will in various degrees, depending on the relative enzyme activity of the different C variants, phosphorylate the CREB protein. Phosphorylated CREB protein binds to the CRE-element and

induces the transcription of luciferase. By using this assay we would be able to determine the intracellular activity of the different C subunits with and without the exon 4.

293T cells were co-transfected with the CRE-luc reporter plasmid, the β-galactosidase control plasmid and one of the following expression vectors; Cα1-HA, Cα1-HAΔ4, Cβ1-HA, Cβ1-HAΔ4, Cβ1, Cβ1Δ4, Cβ3ab or Cβ3abΔ4. Expression of all isoforms was confirmed by Western blotting using anti-Cmono (fig. 4.11, panel A), showing lower expression levels of the Cβ1Δ4 and Cβ3abΔ4 variants. Measuring the luciferase activity in the transfected cells demonstrated that all the normal C subunits induced luciferase activity far above mock levels (cells transfected with CRE-luc vector and β-galactosidase control plasmid only) (fig. 4.11, panel B). In contrast, none of the C variants lacking exon 4 were able to induce luciferase activity above background (mock) level. The luciferase activity of each sample was adjusted according to β-galactosidase-indicated transfection efficiency, and there seemed to be no differences in acticity between Cα1-HAΔ4 and Cβ1-HAΔ4 expressed at a high level and Cβ1Δ4 and Cβ3abΔ4 expressed at a low level. The results from the luciferase reporter assay supported the results from the kinase assays, and we therefore concluded that the PKA CΔ4 subunit variants were catalytically inactive.

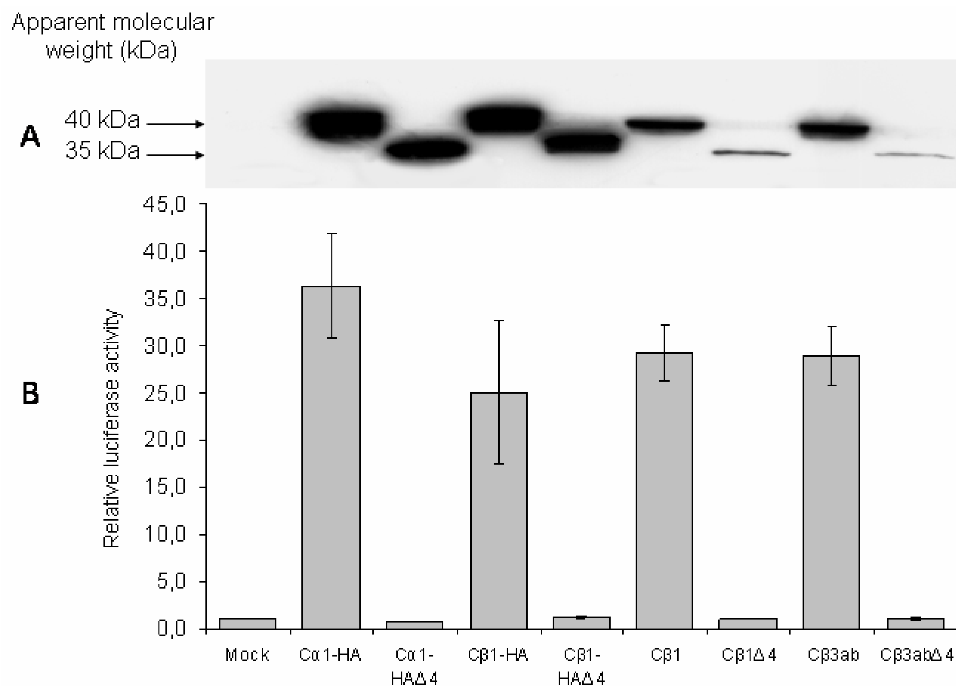

Figure 4.11 C subunits lacking the exon 4 sequence do not induce CRE-dependent luciferase activity

A) 293T cells were co-transfected with a CRE-luc reporter plasmid, a β-galactosidase control plasmid and one of the following expression vectors; Cα1-HA, Cα1-HAΔ4, Cβ1-HA, Cβ1-HAΔ4, Cβ1, Cβ1Δ4, Cβ3ab or Cβ3abΔ4. Mock samples were transfected with CRE-luc reporter plasmid and β-galactosidase control plasmid only. Cells lysates were analysed for the expression of anti-Cmono immunoreactive proteins by separating the cell extracts by SDS-PAGE (12.5% gels) and immunoblotting. This revealed that all C variants were expressed at detectable levels and with the expected relative sizes (kDa). B) Cell lysates as shown under A) were monitored for luciferase activity which is compared to background activity in mock transfected cells. The results presented represent the average from three representative experiments, +/- the standard error of the mean (SEM).

4.3 Characterisation of the CβΔ4 protein interaction with the R subunit

4.3.1 Cyclic AMP independent interaction of CβΔ4 variants with RIIα

All known C subunits of PKA bind to and are inactivated by the R subunits RI and RII. This interaction is regulated by cAMP (Skålhegg & Taskén, 2000). To further characterise the features associated with the CβΔ4 variants, we tested their ability to bind to the R subunit RIIα and the effect cAMP would have on this interaction.

293T cells were co-transfected with RIIα and either Cβ1-HA or Cβ1-HAΔ4, or the untagged C subunits Cβ1, Cβ1Δ4, Cβ3ab or Cβ3abΔ4. Cell lysates were immunoprecipitated with anti-RIIα followed by Western blot analysis using anti-Cmono. This showed that all the normal Cβ splice variants and the splice variants lacking the exon 4 were precipitated by anti-RIIα (fig. 4.12, blot A, B and C, lanes 1 and 9), implying that the R and C subunits co-localize on the same PKA holoenzyme. To test if the interactions were cAMP sensitive, the immunoprecipitates were incubated with 1 mM cAMP and both the pellet (P) and the supernatant (S) were analysed by immunoblotting. As expected, we found that cAMP released the normal Cβ variants from RIIα since the C subunits were found in the supernatant after cAMP treatment (fig. 4.12, blot A, B and C, lane 4). By contrast, the CβΔ4 variants were not released by 1 mM cAMP as Cβ1-HAΔ4, Cβ1Δ4 and Cβ3abΔ4 were all detected in the pellet (fig. 4.12, blot A, B and C, lane 11). Control experiments were performed by immunoprecipitating with irrelevant IgG and by washing the pellets both with and without cAMP (fig. 4.12, lanes 5-8 and 13-16). The experiments showed that the different C subunits were not released from the pellet in the absence of cAMP (fig. 4.12, blot A, B and C, lanes 1 and 9). In blot C, lane 3, a Cβ3ab immunoreactive band is still present in the pellet sample, implying incomplete cAMP-release of Cβ3ab from the pellet. This may be caused by a high Cβ3ab protein level combined with suboptimal wash of the pellet.

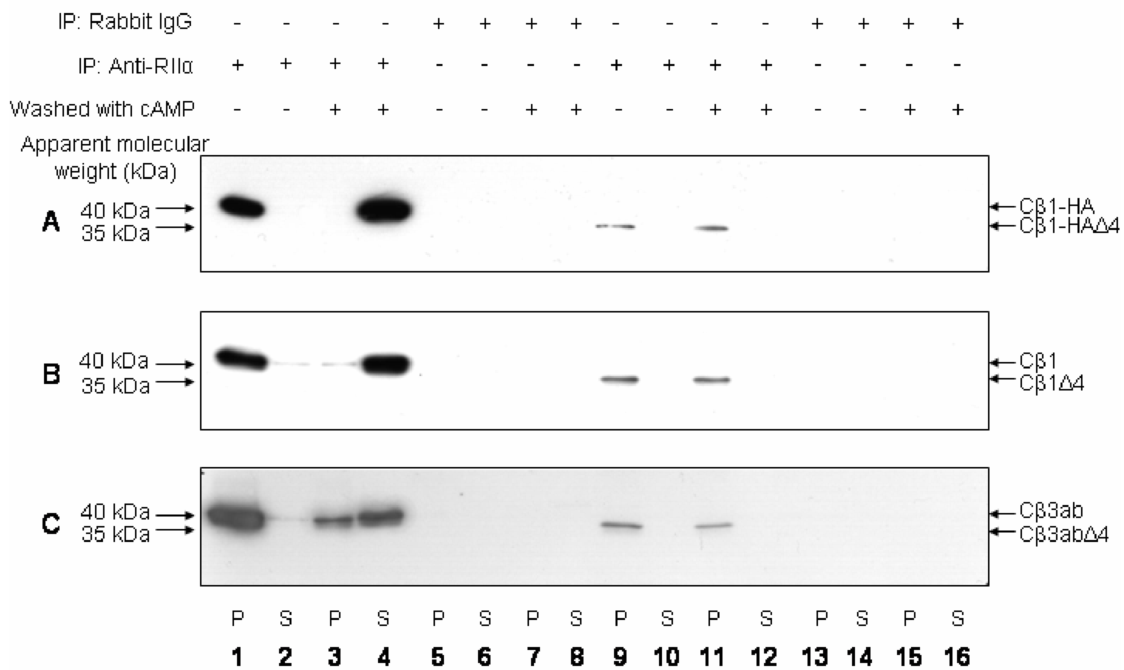

Figure 4.12 Cyclic AMP dependent and independent interactions of Cβ subunits with RIIα in 293T cells

293T cells were co-transfected with RIIα and either Cβ1-HA, Cβ1-HAΔ4, Cβ1, Cβ1Δ4, Cβ3ab or Cβ3abΔ4 and lysed. Cell extracts were immunoprecipitated with anti-RIIα antibody (lanes 1-4 and 9-12) and rabbit IgG (lanes 5-8 and 13-16). Immunoprecipitated proteins were treated with (+) or without (-) 1 mM cAMP. The pellets (P) and supernatants (S) were subjected to SDS-PAGE (12.5% gels) and Western blotting using anti-Cmono. A) Results from Cβ1-HA and Cβ1-HAΔ4 co-transfected with RIIα. B) Results from Cβ1 and Cβ1Δ4 co-transfected with RIIα. C) Results from Cβ3ab and Cb3abΔ4 co-transfected with RIIα. Note that none of the Δ4 variants are released from RIIα by 1 mM cAMP. Arrows on the left indicate apparent molecular weight and arrows on the right indicate C subunit identity.

5. Discussion

In the present study, we have identified and characterised several novel PKA Cβ subunits that lack the sequence encoded by the exon 4 in the Cβ gene. All the novel CβΔ4 variants were identified in NT2 cells differentiated to neuronal NT2-N cells by RA. CβΔ4 variants were also expressed in human brain. Interestingly, human PBL, which express Cβ1 and Cβ2, were shown to be devoid of detectable levels of CβΔ4 despite using a novel and sensitive method developed in the present work to detect low levels of CβΔ4 mRNA. Together these results imply that splicing out the exon 4 of the Cβ subunit gene is a nerve cell-specific event. Moreover, the CΔ4 variants were shown to completely lack catalytic activity in that they were incapable of phosphorylating the well-known PKA substrates kemptide and histone, and did not induce the expression of a CRE-regulated luciferase reporter gene. Despite the lack of catalytic activity, the CβΔ4 variants were shown to bind to the R subunit, but in a cAMP-insensitive fashion.

5.1 Splicing of the exon 4 in the Cβ gene is a phenomenon associated with the Cβ splice variants in nerve cells

The Ntera2cl.D1 (NT2) cell line is a teratocarcinoma cell line that differentiates along a neuro-ectodermal lineage after exposure to RA. RA treatment combined with mitotic inhibitors in the presence of adhesion matrices yields >95% pure cultures of neuronal cells, NT2-N cells. The NT2-N cells exhibit a stable and irreversible neuronal phenotype, with lack of mitotic activity and phenotypic reversion. A variety of neuronal markers are expressed, for instance cytoskeletal proteins, secretory markers and surface markers (Pleasure *et al*., 1992). Morphologically, the NT2-N cells resemble primary neuronal cultures from rodents and they elaborate processes that differentiate into axons and dendrites. They also have specific characteristics which indicate that they are neurones of the central and not the peripheral nervous system (Pleasure *et al.,* 1992). Differentiating and fully differentiated NT2-N cells

therefore represent an excellent model system for studies of human neurones. The fact that these cells also express all the known splice variants of the Cβ isoform upon RA-dependent differentiation (Kvissel *et al.*, 2004) makes them an interesting system for studying PKA-dependent effects on nerve cell differentiation and function.

During experiments performed in our group, it appeared that one of the NT2-N expressed Cβ products seemed to lack a sequence corresponding to the exon 4 in the Cβ gene. To further study this phenomenon and to demonstrate whether such splice variant mRNAs really were expressed, PCR using Cβ splice variant specific as well as Cβ common primers was applied. In this way we were able to identify six Cβ variants lacking the exon 4 sequence. During these experiments we also tested, using the same techniques, if any Cα variants lack the exon 4. We were unable to identify any Δ4 variants transcribed from the Cα gene, suggesting that splicing out the exon 4 is a phenomenon specific to the Cβ gene.

As RA-differentiated NT2-N cells are considered to be neurones, we tested if CβΔ4 variants were expressed in the brain. Indeed, we were able to identify, using the Cβ common primers, that CβΔ4 variants are expressed in the human brain. This was not the case for human PBL, which also express Cβ1 and Cβ2 (Ørstavik *et al.*, 2001). However, one should notice that the levels of Cβ expression in leukocytes are significantly lower than in the brain. The fact that the CβΔ4 variants were expressed at low levels may imply that the CβΔ4 variants in leukocytes were expressed at non-detectable levels. This was the motivation for the development of a method which enabled us to improve the sensitivity for detecting CβΔ4 variants. As explained in detail in the result section, this method was developed to enhance the relative amount of Cβ fragments lacking the exon 4 sequence. The method takes advantage of the fact that the restriction enzyme SspI has a unique recognition site in the Cβ exon 4 sequence. A first PCR amplification round with the Cβ common primers and a low cycle number would supposedly favour the amplification of Cβ variants with the exon 4 sequence as these are in surplus. After digestion with SspI, a second PCR round applying the same primers would favour the amplification of CβΔ4 variants.

This was a promising strategy, but on a few occasions we experienced incomplete digestion of the exon 4-containing species. The incomplete digestion, combined with the high sensitivity of the PCR method, resulted in the sustained presence of the upper Cβ band representing Cβ variants containing the exon 4 sequence. The phenomenon is illustrated in figure 4.7, lane 9, where the upper band is weakly apparent even after SspI digestion. Re-digestion of the first PCR reaction was performed on a few occasions and can, if necessary, be used to improve the results. Cases of incomplete digestion may be considered to be a problem with the screening method; however, we do not consider this to be serious. Even in cases of incomplete digestion, CβΔ4 variants are more easily detected, because, after all, the level of Cβ species containing the exon 4 is reduced.

Interestingly, when we applied the screening method on human PBL, we were still not able to detect any CβΔ4 variants. Despite this, we cannot completely rule out the possibility of the expression of a CβΔ4 variant in leukocytes. Taken together, our results suggest that splicing out the PKA Cβ exon 4 may be a nerve cell specific phenomenon that does not take place in other cells. It would be of great interest to apply the screening method to cDNA synthesised from RNA isolated from different areas of the brain and other non-neuronal cells than PBL, in order to investigate if there is a splice variant specific expression pattern.

5.2 The novel Cβ splice variants lacking the exon 4 encoded sequence are catalytically inactive and form cAMP-insensitive PKA holoenzymes

Positioning the exon 4 into the previously published Cα 3D structure (Knighton *et al.*, 1991) and rotating the model 360° demonstrated that the exon 4 encoded sequence is a crucial component of the catalytic cleft in the C subunits. This is most probably the case for all known C subunits. Consequently we would expect that any changes in activity monitored for one of the CβΔ4 variants would be evident for all the Cβ variants lacking this sequence. Indeed, all *in vitro* testing of C subunits lacking the

exon 4 revealed that they were incapable of phosphorylation the two well-characterised PKA substrates kemptide and histone (Zhang *et al*., 2004). In addition, none of the CΔ4 variants were able to induce a CRE-regulated promoter as tested by the luciferase reporter assay which is an assay that may be considered a more *in vivo* test system for PKA catalytic activity. Taken together with the position of the exon 4 in the globular protein, our results suggest that lack of the exon 4 encoded sequence induces a structural change of the catalytic cleft, rendering these C variants inactive. Interestingly, we observed that Cα1-HA displayed a substrate preference, showing almost no phosphotransferase activity against histone. It has previously been reported that for Cγ, histone is a better substrate than kemptide (Beebe *et al*., 1992) so it is probable that such differences exist. This study was not aimed at elucidating the differences in enzyme kinetics of the various C subunits, and the phenomenon was not studied further.

To further characterise the CβΔ4 variants, we also tested their ability to associate with the R subunit to form PKA holoenzymes. In our immunoprecipitation experiments, we demonstrated that Cβ1-HAΔ4, Cβ1Δ4 and Cβ3abΔ4 bind to the RIIα subunit. However, this association was insensitive to high levels (1 mM) of cAMP, implying an irregular association of CβΔ4 with the R subunit. The CβΔ4 variants may bind to the R subunit in a fashion that blocks the binding of cAMP, or in a way that leads to high affinity for the R subunit even when cAMP is bound.

In the present study, CβΔ4 and R-interactions were only tested for the RII subunit and should in future studies be tested for the RI subunit as well in order to test if the two R isoforms behave differently towards the CβΔ4 variants. Preliminary results indicate an association between CβΔ4 and RIα as determined by immunoprecipitation (Cecilia Avellan, personal communication). Further investigation of the R-binding characteristics of the CβΔ4 variants requires *in vitro* expressed and purified CβΔ4 and R subunits, and monitoration of the binding and dissociation kinetics. This was considered beyond the scope of this thesis, but it will be investigated in collaboration with professor Stein Ove Døskeland at the University of Bergen.

5.3 The importance of alternative RNA splicing

The human genome is now fully sequenced and the number of protein-coding genes is estimated to approximately 30.000. Humans generate a considerably larger number of proteins than the number of available genes. Post-translational modifications, RNA editing, alternative polyadenylation and multiple start sites of transcription contribute to generating diversity, but alternative splicing is the major mechanism by which this is achieved (Grabowski & Black, 2001).

The regulation of nerve cell differentiation is a complex process and the development of a neurone-specific phenotype, primarily measured by the outgrowth of axons and the formation of functional synapses, must be carefully adjusted. Alternative splicing is an excellent means for diversifying the properties of a protein and can give each splice variant specific and fine-tuned characteristics. Tissue-specific alternative splicing is a well-known feature and is nowhere more evident than in the nervous system (Grabowski & Black, 2001). As already mentioned, alternative splicing occurs especially frequently in the nervous system and the PKA Cβ subunit is a gene that is highly differentially spliced in the N-terminal region, leading to the expression of the neuronal-specific Cβ splice variants Cβ3 and Cβ4 with different exon a, b and c combinations (Ørstavik *et al*., 2001, Kvissel *et al*., 2004).

RA has been reported to change the splicing pattern of the β A4 amyloid protein precursor (APP) splice variants in the RA-induced differentiated neuroblastoma cell line SH-SY5Y (König *et al*., 1990) and it was speculated that RA might induce the expression of specific splicing factors responsible for the alternative splicing of the gene encoding for APP. Another study reported that in the RA-induced F9 teratocarcinoma stem cell line, one of the RA homeobox target genes was alternatively spliced producing one full length and one truncated homeoboxless protein (LaRosas & Gudas, 1988). The relative expression of the truncated protein was induced after RA treatment and there is a possibility that a RA-regulated activity influences the splicing pattern in the mRNAs so that the affinity for additional

splicing sites is increased. The truncated protein was believed to have a potential for regulatory interactions.

A large-scale analysis of protein isoforms arising from alternative splicing shows that disruption of protein domains is less frequent than insertions or deletions of entire domains. Nevertheless, the functional effect of such disruptions, predicted from 3D structure, is often equivalent to removal of an entire domain (Kriventseva *et al.*, 2003), like in our case with the new splice variants. The biological significance of the CβΔ4 splice variants is unknown, but it is interesting that they bind to the R subunit in a cAMP-independent fashion and they may serve as a buffer for the R subunit. To what extent the novel splice variants are expressed as proteins in cells is difficult to estimate and has not been investigated yet. By interpreting the PCR results, we assume that the CβΔ4 variants are expressed at such a low level that it is difficult to detect them by conventional Western blotting. However, a way which potentially could make it possible to detect the CβΔ4 variants would be to make use of the fact that the CβΔ4 variants bind to the R subunit even in the presence of high cAMP-levels. CβΔ4 variants could be immunoprecipitated with anti-R antibody from brain tissues in the presence of high levels of cAMP followed by anti-Cmono immunoblotting. This procedure could make it possible to detect even low levels of CβΔ4 protein expression, but was not applied in this work due to difficulties in obtaining fresh human brain tissue in sufficient quantities. If the CβΔ4 variants are expressed, they may be important modulators of total PKA activity in the cell.

6. Conclusions

The results from this thesis demonstrate that:

1) 6 novel PKA Cβ splice variants are expressed at the mRNA-level in NT2-N cells differentiated by RA, all lacking the exon 4 sequence.

2) The novel splice variants are catalytically inactive against the PKA substrates kemptide and histone and can not induce a CRE-regulated promoter.

3) The novel splice variants bind to the RIIα subunit, but are insensitive to release by 1 mM cAMP.

4) A new screening method can be used to detect low levels of CβΔ4 mRNA and show that the new CβΔ4 variants are expressed in human brain, but probably not in human PBL.

5) Cβ3ab phosphorylates both kemptide and histone and also induces a CRE-regulated promoter.

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