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THE FUNCTIONS AND REGULATION

OF ORNITHINE DECARBOXYLASE

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ACADEMIC DISSERTATION

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ABSTRACT

Ornithine decarboxylase (ODC) regulates the synthesis of polyamines which are involved in many cellular functions e.g. proliferation and differentiation. Due to its critical role, ODC is a tightly regulated enzyme. If the regulation fails, the activity of ODC increases and may even lead to malignant transformation of a cell. Increased ODC activity is found in many common cancers. ODC plays an important role also in the central nervous system (CNS). It is involved in brain morphogenesis and in the regulation of glutamate receptors (especially NMDA-receptors) and the K^+ inward rectifier channels. High ODC activity and polyamine levels are considered to be important in the development of ischemic brain damage and they are implicated in the pathogenesis of Alzheimer's disease (AD). The aim of this study was to investigate the molecular mechanisms by which polyamines and ODC influence cell proliferation and transformation (I). Furthermore, the investigations were focussed at the regulatory factors of the polyamine-ODC system in the CNS (II-IV).

Reorganization of the actin cytoskeleton is a fundamental event during proliferation and cell transformation. It has been implicated that polyamines participate in the regulation of actin cytoskeletal dynamics, although the actual molecular mechanism has remained unsolved. The small G protein, RhoA is a regulator of actin reorganization. Some bacterial toxins are known to covalently link polyamines to RhoA. Polyamination activates RhoA constitutively and facilitates the intrusion of bacteria into cells. The present results demonstrate that polyamination of RhoA takes place also physiologically in cells without bacterial toxins (I). Inhibition of transglutaminase 2 (TG2) abolished the polyamination, thus indicating that TG2 has a catalytic role in the covalent linking of polyamines to RhoA. Studies on synchronized fibroblasts by western blotting with antibodies to RhoA showed that the polyamination of RhoA was most extensive in the cell cycle phases of G2 and mitosis. Furthermore, analysis by flow-cytometry, revealed that efficient progression in the cell cycle of Jurkat cells depended on the intact function of TG2 and the polyamination of RhoA. When cells devoid of catalytically active ODC were transfected with a mutant form of ODC, in which the membrane translocation PHOX-motif was deleted, ODC activity was detected in the cytoplasmic fraction only.

Neither was any RhoA found in the membrane fraction of these cells whereas immunofluorescence staining showed a diffuse cytoplasmic distribution of RhoA. These results indicate that ODC not only regulates the activity of RhoA by the production of polyamines but also influences its intracellular distribution.

A homolog of ODC was cloned from a human brain cDNA library. The novel protein was nevertheless devoid of ODC catalytic activity (II). It was subsequently found to be a novel inducer of ODC activity and polyamine synthesis, called antizyme inhibitor 2 (AZIN2). Transcripts of human *AZIN2* were most abundant in brain and testis as detected by mRNA dot blot and *in situ* hybridization (II-IV). Ten alternatively spliced forms of AZIN2 were sequenced (II, IV). The distribution of the protein was investigated by immunohistochemistry in order to elucidate the functional role of AZIN2. Human brain and testis were studied more closely. Expression of AZIN2 was detected in steroid hormone-producing cells of the gonads: in Leydig cells of the testis and in luteal cells of the ovary (III). In human brain, AZIN2 was localized along the axons covering tracts from all areas of the CNS studied. A vesicular or granular distribution of AZIN2 was also detected in the somas of cortical pyramidal cells (IV). Reverse transcriptase PCR and western blotting revealed the expression of different splicing variants of AZIN2 in the white as well as grey matter of the brain (IV). Although the distribution of AZIN2 in pyramidal cells differed spatially, double immunofluorescence revealed a partial co-localization with the ubiquitously expressed NMDA-type glutamate receptors (NMDAR). Polyamines, ODC and NMDAR are implicated in the pathogenesis of AD. An accumulation of also AZIN2 was detected in specimens of AD brains (IV). This increased expression of AZIN2 was specific for AD and was not found in brains with other neurodegenerative diseases including CADASIL or dementia with Lewy bodies.

In conclusion, the results indicate that covalent attachment of polyamines by transglutaminase is a physiological means of regulating the activity of RhoA. The translocation of RhoA to the plasma membrane, where it exerts its activity is dependent on the presence of catalytically active ODC. As the overactivity of ODC and RhoA are implicated in cell transformation, the results provide a mechanistic explanation of the interrelationship between the polyamine metabolism and the reorganization of the actin cytoskeleton occurring in cancer cells.

In addition, a novel regulator of polyamine synthesis, AZIN2 was cloned and characterized. The distribution of AZIN2 is more restricted in human tissues than its functional and structural homolog antizyme inhibitor 1 (AZIN1). The accumulation of AZIN2 in vesicle-like formations along the axons and beneath the plasma membrane of neurons as well as in steroid hormone producing Leydig cells and luteal cells of the gonads implies that AZIN2 plays a role in secretion and vesicle trafficking.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on four original publications and previously unpublished data. The original articles are referred to in the text by their Roman numerals.

I. Mäkitie LT, Kanerva K, Andersson LC: Ornithine decarboxylase regulates the activation and localization of RhoA by polyamination. *Experimental Cell research* 315 (6): 1008-1014, 2009.

II. Pitkänen LT, Heiskala M, Andersson LC: Expression of a novel human ornithine decarboxylase-like protein in the central nervous system and testes. *Biochemical and Biophysical Research Communications* 287 (5): 1051-1057, 2001.

III. Mäkitie LT, Kanerva K, Sankila A, Andersson LC: High expression of antizyme inhibitor 2, an activator of ornithine decarboxylase in steroidogenic cells of human gonads. *Histochemistry and cell biology* 132 (6): 633-638, 2009.

I. Mäkitie LT, Kanerva K, Polvikoski T, Paetau A, Andersson LC: Brain Neurons Express Ornithine Decarboxylase-Activating Antizyme Inhibitor 2 with Accumulation in Alzheimer's Disease. *Brain Pathology* 2009. DOI 10.1111/j.1750.2009.00334.x

ABBREVIATIONS

AD	Alzheimer's disease
ADC	arginine decarboxylase
adomet-DC	S-adenosylmethionine decarboxylase
AMPA	alfa-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AZ	antizyme
AZIN	antizyme inhibitor
Ca^{2+}	calcium ion
CA1-3	subdivisions 1-3 of Ammon's horn / *cornu Ammonis*
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
cdk	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary cells
CNS	central nervous system
DFMO	α-difluoromethylornithine
DLB	dementia with Lewy bodies
DNA	deoxyribonucleic acid
egr-1	early growth response 1
eIF5A	eukaryotic initiation factor 5A
ER	endoplasmic reticulum
ERK	extracellular-signal-regulated kinase
EST	expressed sequence tag
FOS	FBJ murine osteosarcoma viral oncogene homolog
G1	a growth phase of the cell cycle
G2	final subphase of interphase
GAP	GTPase activating protein
GDI	guanine nucleotide-dissociation inhibitors
GDP	guanosine diphosphate
GEF	guanine nucleotide-exchange factor
GTP	guanosine triphosphate
GTPase	enzyme hydrolyzing guanosine triphosphate
JunD	jun D proto-oncogene
Kir	K^+ inward rectifier channel
LH	luteinizing hormone
LIM kinase	a serine kinase regulating cytoskeleton
MAPK	mitogen-activated protein kinase
mDia	mammalian diaphanous
MDC	monodansylcadaverine
MEK kinase	MAPK/ERK kinase kinase
MLC	myosin light chain
mRNA	messenger ribonucleic acid
MYC	myelocytomatosis oncogene
NAP	nuclear aggregates of polyamines
NIH 3T3	immortalized mouse embryonic fibroblast cell line
NMDAR	N-methyl D-aspartate type glutamate-receptor
ODC	ornithine decarboxylase
ODCp	ornithine decarboxylase paralog
PHOX	phagocyte oxydase motif
P0	postnatal day 0
p53	tumor protein p53
PEST	domain rich in proline, glutamic acid, serine, threonine
PI3K	phosphoinositide-3-kinase
PRE	polyamine response element
PSV	polyamine sequestering vesicle
Ras	rat sarcoma oncogene
RBL-1	rat basophil cell line
RhoA	ras homolog gene family, member A
RNA	ribonucleic acid
ROCK	Rho kinase
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
RT	room temperature
siRNA	small interfering RNA
Smurf	E3 ubiquitin ligase
Src	Rous sarcoma oncogene
SSAT	spermidine/spermine *N1*-acetyltransferase
TG2	transglutaminase 2 (tissuetransglutaminase)
TGF-β	transforming growth factor β
tRNA	transfer ribonucleic acid
Ty1	Transposon yeast 1 transposable element

INTRODUCTION

Polyamines are small cationic molecules found in all eu- and prokaryotic cells. These molecules bind to anionic molecules such as RNA, DNA, proteins, and phospholipids. They induce changes in the electrostatic status leading to modifications of the macromolecules. Binding of polyamines typically increases the stability of a macromolecule by creating a more compact form and providing protection against endo- and exogenous hazards including irradiation and reactive oxygen species. A majority of the high millimolar concentration of polyamines is constantly attached to nucleic acids. Polyamines also function as signalling molecules especially in mitogenic signalling cascades. Their binding and detachment, especially to proteins, is rapidly regulated by e.g. transglutaminases.
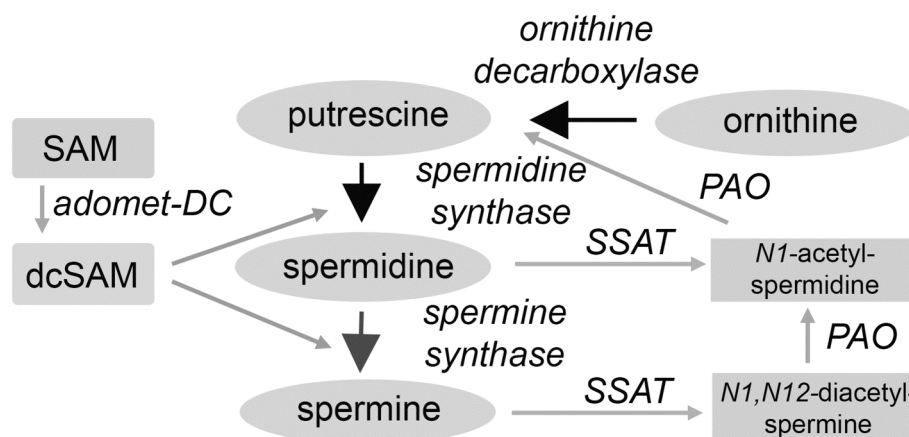

Figure 1. *The metabolic pathway of mammalian polyamines. SAM - S-adenosylmethionine, adomet-DC - S-adenosylmethionine decarboxylase, SSAT - spermidine/spermine-N^1 -acetyl-transferase, PAO - polyamine oxidase.*

The metabolism of polyamines is regulated by multiple enzymes which in turn are tightly controlled at the transcriptional, translational and post-translational levels by other regulators. ODC is the first rate-limiting enzyme in polyamine synthesis and because of its essential role is being monitored constantly. The half-life of ODC is very short, only 10–20 minutes in eukaryotes, emphasizing the importance of its regulation. ODC is bound to its inhibitor antizyme (AZ) which targets ODC to proteasomal degradation independently of ubiquitination. When the concentration of polyamines falls, ODC is synthesized *de novo* and also released from the AZ-complex

by antizyme inhibitor. Antizyme inhibitor (AZIN) is a protein homologous to ODC but devoid of decarboxylating activity. Due to its homology it replaces ODC from AZ and leads to increased polyamine synthesis. AZ and AZIN regulate also the cellular uptake of polyamines.

Polyamines participate in the regulation of numerous cellular functions of which proliferation is the most studied. Cells are unable to proliferate without polyamines. These are needed for the regulation of cyclins and cyclin dependent kinases (cdk), the reorganization of the cytoskeleton, and as a part of ras-, mitogen-activated protein kinase (MAPK), and src-signalling cascades. Lack of polyamines halts the cell cycle in G1, but also a constantly upregulated polyamine synthesis disturbs the progress of the cell cycle. Polyamines are considered fundamental also in differentiation and morphogenesis. Transgenic mice with increased activity of ODC and content of polyamines in the testis are infertile and show impaired spermatogenesis. Enhanced catabolism of spermidine and spermine in SSAT transgenic mice leads to female infertility with hypoplasia of the uterus and absence of the developing follicles and corpus luteum in the ovary. Homozygous deletion of the *ODC* gene (knock-out) in mice is embryonally lethal. The concentration of polyamines is invariably monitored and regulated not only by *de novo* synthesis and catabolism but also by active uptake and efflux from the extracellular space. Polyamines are obtained from alimentary sources and they are produced by the intestinal bacterial flora.

The concentration of polyamines is elevated in several human cancers. The activity of ODC is constitutively increased e.g. in cancers of the prostate, breast, and colon. Furthermore, overexpression of ODC via transfection leads to malignant transformation of NIH3T3 fibroblasts indicating an oncogenic role for ODC. Such ODC-overexpressing cells are tumorigenic in nude mice. Polyamine analogs and ODC inhibitors have been studied and used as chemotherapeutics and adjuvants in the treatment of cancer. However, the ultimate molecular mechanisms by which polyamines and their regulators influence the cell transformation are not known. The participation of polyamines in the regulation of the cell cycle, mitogenic signalling, and cytoskeletal reorganization is considered to be essential.

In addition to transformation and cancer, polyamines are connected to pathologies of the brain. Elevated ODC activity and polyamine levels are measured from brains affected by Alzheimer's disease. Furthermore, in ischemic lesions polyamines accumulate in neurons of the penumbra. However, their role in the pathogenesis is still unclear. Whether polyamines are neuroprotective or neurotoxic is still controversial, but the assumption that they function as scavengers of free oxygen radicals refers to a protective role. In normal brain, polyamines participate in neuronal signalling. They contribute to signalling by regulating glutamate receptors including NMDA receptors, which are connected to memory and learning. The formation of action potentials is controlled by K^+ inward rectifying channels which are gated by polyamines.

REVIEW OF THE LITERATURE

1. Functions of polyamines and ODC

1.1. Polyamines bind nucleic acids

Polyamines are small, positively charged molecules that exist in all living organisms. In mammals, the predominant polyamines are spermidine and spermine and their diamine precursor, putrescine. Due to their cationic nature in physiological pH, they bind cellular molecules of opposite charge: RNA, DNA, nucleotide triphosphates, phospholipids, and proteins (Igarashi K et al. 1982, reviewed in Bachrach U 2005). Only 2–15 % of polyamines are free in the cytoplasm, whereas the majority are bound, mainly to RNA (Igarashi K, et al. 2000). *De novo* synthesized polyamines are rapidly recruited to anionic compounds in a cell: free putrescine is estimated to vanish in 2h, spermidine in one day, and 60% of spermine is bound in four days (McCormack SA, et al. 1993).

Since most of the intracellular spermidine and spermine is bound to RNA, this interaction is considered to mediate the majority of the functions of polyamines (Igarashi K et al. 2000). Polyamines bind both non-coding transfer and ribosomal RNAs and message RNA, consequently leading to changes in their secondary structure. tRNA is stabilized and protected from degradation by polyamines, whereas the phosphate-groups of rRNA are neutralized by spermidine and spermine, enabling ribosomal aggregation (Quigley GJ et al. 1978, Erdmann VA et al. 1968, Zillig W et al. 1959, Xaplanteri MA et al. 2005, Venkiteswaran S et al. 2005). Polyamine-mediated changes usually stabilize the structures and stimulate the efficacy and fidelity of translation (Igarashi K et al. 1974, Igarashi K et al. 1979, Jelenc PC et al. 1979). Due to its higher charge compared to spermidine or putrescine, spermine stabilizes and aggregates anionic molecules more potently.

In prokaryotic cells, translational frameshifting in mRNA reading is a common regulative mechanism. Frameshifting in eukaryotes, on the other hand, is a rare found only in the translation of antizyme that is expressed also in mammals and in the Ty1 transposable element in yeast. Antizyme (AZ) is a negative regulator of ODC activity

and polyamine transport. Its translation is reinforced by elevation in polyamine concentration. Polyamines induce +1 shift of the reading frame in ribosomes leading to the formation of active, full-length antizyme-protein, which further decreases the concentration of polyamines (Matsufuji S et al. 1995). mRNA of AZ contains 5′-element that senses the concentration of polyamines. During translation in ribosomes, polyamines might also slow down the reading facilitating frame-shifting (Petros LM, et al. 2005). Thus, polyamines display negative feedback regulation via antizyme. The archaic regulation of expression by ribosomal frame-shifting underscores the fundamental nature of the polyamine metabolism.

Elevated levels of polyamines stimulate the expression of certain transcription factors related to the mitogen-activated protein kinase (MAPK) pathway and activated by mitogenic stimulus (*c-fos, c-myc*) (Patel AR et al. 1997). Instead, depletion of polyamines provided by the administration of the ODC inhibitor, DFMO (α-difluoromethylornithine) decreases the mRNA levels of *c-fos, c-myc, c-jun,* and *Egr-1* (Wang JY et al. 1993, Li L et al. 2001, Stephenson AH et al. 2004). Polyamine depletion halts progression of the cell cycle. Decreased levels of polyamines also stabilize the transcripts of certain growth inhibitory genes, e.g. *p53, TGF-β,* and *junD* (Patel AR et al. 1997, Li L et al. 2002, Li L et al. 1999). The mechanism by which polyamines regulate the expression of various genes is not fully established, but the polyamine response element (PRE) has been recognized in the untranslated region of certain genes, e.g. spermidine-spermine-N^1-acetyltransferase (SSAT) (Wang Y et al. 1998, Stephenson AH et al. 2006). SSAT regulates the levels of polyamines by converting spermine to spermidine and spermidine to putrescine, and thereby contributes to the catabolism and circulation of polyamines.

Polyamines bind to phosphate groups of double-stranded and even triplex and quadruplex DNA, forming intra- and interchain bridges which stabilize the structure (Raspaud E et al. 1998, Saminathan M et al. 1999). Furthermore, polyamines regulate the conformational changes of DNA between A, B, and Z forms (Bryson K et al. 2000, Hasan R et al. 1995). Nuclear aggregates of polyamines (NAP) are concentrates of phosphate anions and polyamines which bind DNA and further stabilize it, but also enable the duplication of DNA during proliferation. The packing of DNA is

reinforced by polyamines in nucleosomes which stabilizes the structure and protects it from damage induced by e.g. radiation, reactive oxygen species, or enzymatic digestion (Snyder RD 1989a, Khan AU et al. 1992, Pedreno E et al. 2005, Snyder RD 1989b). Depletion of polyamines, instead, prolongs the cell cycle causes chromosomal aberrations, e.g. chromosome elongation, unpacking, fragmentation, and breaks. Polyamines regulate histone-modifying protein complexes leading to changes in the acetylation of histones (Hobbs CA et al. 2002). Acetylation of histones enables the binding of promoting factors, e.g. transcription factors, to selected genes. Furthermore, the binding of polyamines to DNA provokes its bending, which is of importance in the initiation of transcription (Peng HF, et al. 2000). DNA bending enables the binding of RNA polymerases and transcription factors thus promoting gene expression.

The binding of polyamines to nucleic acids serves a dual function: 1) the structure of DNA and RNA is stabilized through packing and protected from degradation; 2) polyamines respond to mitogenic stimuli by enhancing proliferation. This latter task requires a higher concentration of free, rapidly mobilized polyamines whereas in nucleic acid stabilization the binding is more stabile. Since depletion as well as excess of polyamines can have a similar effect on cell behaviour, e.g. the inhibition of proliferation and the induction of apoptosis, polyamines have been suggested to dynamically regulate the proliferative state of a cell (for a review, see Thomas T et al. 2001). Electrostatic forces between cationic polyamines and anionic nucleic acids are believed to be responsible for the interactions. However, structural aspects, such as the flexibility and rod-like structure of polyamines and the sequence-specificity of nucleic acids also play a role in the binding.

1.2. Interaction of polyamines with proteins

Transglutaminases (TG) are widely distributed bifunctional enzymes, which also have non-catalytic functions (for reviews, see Griffin M et al. 2002, Lorand L et al. 2003). These enzymes catalyze the hydrolyzation of GTP and post-translational modification of proteins, the reactions of which are competitive and partly reversible. TGs induce transamidation of amine compounds to γ-glutaminyl residues of proteins. Polyamines

are the major source of amines (Piacentini M et al. 1988, Folk JE et al. 1980), but also other primary amines are utilized in the covalent linkages (Guilluy C et al. 2007). Incorporation of amines increases the net charge of a protein, which changes conformation, solubility, stability, and interactions with other macromolecules. Transamidation of proteins is considered a biologically important post-translational modification. TG also catalyzes protein-protein cross-linking which triggers many physiological reactions of polymerization and aggregation, e.g. the clotting of blood and semen. Increased transglutaminase activity has been detected in many diseases, including Alzheimer's disease and Huntington disease (Tucholski J et al. 1999, Jeitner TM et al. 2008, Muma NA 2007). Post-translational modifications catalyzed by TGs, including transamidation, esterification, and hydrolysis require high concentration of Ca^{2+}. This can be achieved only in pathological states in which the intracellular Ca^{2+}-storages are released, or by an influx of extracellular Ca^{2+} (Smethurst PA et al. 1996). In addition, transglutaminases have functions which are unrelated to their Ca^{2+}-dependent enzymatic reactions: they modify the extracellular matrix and cellular structures via interactions with the cytoskeleton, adhesions, and integrins; they also participate in signal transduction (Janiak A et al. 2006, Mian S et al. 1995, Gentile V et al. 1992).

TG2 (tissueTG) is a ubiquitously expressed isoform localized in the cytoplasm but found also in the plasma membrane and nucleus (Lesort M et al. 1998, Slife CW et al. 1985). TG2 has been found to link polyamines to (i.e. polyaminate) the G protein, RhoA at its glutamine 63. This polyamination prevents the GTPase activity of RhoA, sustaining it in a constitutively active, GTP-bound form (Flatau G et al. 1997). Certain bacterial toxins function as transglutaminases and activate RhoA by polyamination. Since RhoA is a regulator of the actin cytoskeleton, this provokes the formation of stress fibers and focal adhesions (Masuda M et al. 2000). Polyamination of RhoA has also been implicated in neurite outgrowth and neuronal differentiation (Singh US et al. 2003). The migration of epithelial cells during intestinal restitution has also been demonstrated to be dependent on the concentrations of polyamines and Ca^{2+} and the activity of Rho proteins (Rao JN et al. 2001, Ray RM et al. 2003).

A specific example of polyamine-dependent protein modification is the incorporation of hypusine to eukaryotic initiation factor 5A (eIF5A). A 4-aminobutyl moiety of

spermidine is transferred to eIF5A and further modified to hypusine (Cooper HL et al. 1983). The function of eIF5A is dependent on hypusinylation, and the lack of spermidine inhibits hypusinylation and arrests cell growth (Schnier J et al. 1991, Park MH, et al. 1993). The role of hypusine in cell proliferation is emphasized by the finding that the generation of hypusine is significantly elevated in NIH 3T3 cells transformed by the ras oncogene (Chen ZP et al. 1997). Initially, eIF5A was considered to be an initiator of transcription, but later it was found to play a role in the regulation of mRNA degradation (Zuk D et al. 1998).

The regulation of ion channels by polyamines is of physiological importance in protein-polyamine interaction. Small, cationic polyamines existing both intra- and extracellularly gate strong K^+ inward rectifier channels (Kir) and α-amino-3-hydroxy-4-methyl-5-isoxazolepropionic acid- (AMPA), kainate-, and N-methyl-D-aspartate (NMDA)-type glutamate channels. Polyamines permeate into the channel pore and block the flow of ions through the Kir channels depending on the voltage. In the glutamate receptors, the attachment of polyamines to specific binding sites modulates the electrostatic environment and thus influences the binding capacity of the ligands (reviewed in Williams K 1997). Kir channels allow the inward flow of K^+ under a negative membrane potential (Nichols CG et al. 1997). When depolarization abolishes the voltage difference, spermine and spermidine block the channel causing a relative decrease in intracellular $[K^+]$, which in turn potentiates the amplitude of the action potential (Lopatin AN et al. 1994, Fakler B et al. 1994). Small depolarization waves do not induce the block, but an inflow of K^+ rather continues and counteracts the initiation of depolarization. Kir-channels thus set the threshold for the action potential and stabilize the membrane. They also amplify the depolarization signals that are strong enough to provoke action potentials. Depletion of spermine/spermidine after the inhibition of S-adenosylmethionine decarboxylase or ODC reduces the inward rectification in RBL-1 and CHO cells, respectively (Bianchi L et al. 1996, Shyng SL et al. 1996). A similar reduction in inward rectification was observed in a Gyro-mouse with deleted spermine synthase gene and thus lacking spermine (Lopatin AN et al. 2000). In cardiac myocytes, the excitability is reduced after a decrease in polyamine concentration, suggesting that it might serve as a potential target in the treatment of arrhythmia (Nichols CG, et al. 1996). Kir channels function in various cells, but the

subtypes of channels that are regulated by polyamines reside predominantly in the brain, heart, and skeletal muscle (Oliver D et al. 2000).

The NMDA-, AMPA-, and kainate receptors respond to a presynaptic release of neurotransmitters by creating excitatory postsynaptic potentials (epsps) and depolarization. NMDA-type glutamate receptors allow the intracellular flow of Na^+ and Ca^{2+} upon ligand, i.e. glutamate and glycine, binding (McBain CJ et al. 1994). The extracellular portion of the receptor contains at least two polyamine-binding sites in addition to a binding site in the channel pore to which polyamines have access from both intra- and extracellular sides. The binding of spermine and spermidine to the receptor enhances the binding of the co-ligand, glycine, and increases the probability of the channel to open independently of glycine (Ransom RW et al. 1990). This leads to potentiation of the signalling (Benveniste M et al. 1993). Quite the contrary, polyamines can inhibit the flow of ions by blocking the channel pore (Araneda RC et al. 1999). They also decrease the affinity of glutamate to its receptor (Williams K et al. 1994). Physiologically the most relevant mechanisms of action are considered to be the glycine-independent activation of channel opening and the attenuation of the glutamate-affinity (Williams K 1997). Thus, polyamines strengthen the NMDA response to activation signalling by affecting the amplitude and by decreasing the length of the response. This kind of vigorous, but rapidly silenced signalling is considered fundamental in long-term potentiation and depression, which are pivotal for memory and learning (MacDonald JF et al. 2007, Grosshans DR et al. 2002). Different subtypes of this receptor vary in their ability to bind polyamines, and also in their response to polyamine binding (Williams K et al. 1994, Durand GM et al. 1993). Neuronal stimulation alters the composition of receptors and new receptors are recruited to the synaptic density from mobile transport packets and extrasynaptic membrane areas (Lau CG et al. 2007, Quinlan EM et al. 1999, Ying Z et al. 1998, Washbourne P et al. 2004). Subunits of receptors with a high affinity for spermine are expressed embryonally and neonatally, indicating a role for polyamines in morphogenesis, e.g. synaptogenesis and axonogenesis (Zhong J et al. 1995).

AMPA- and kainate-type glutamate receptors mediate fast synaptic transmission at excitatory synapses by permeating Na^+ and occasionally also Ca^{2+}. Subtypes passing Ca^{2+} are voltage-dependently blocked by polyamines, establishing inward

rectification. Such receptors are expressed in the fetal and neonatal central nervous system (Shin J et al. 2005, Koh DS et al. 1995, Bernard A et al. 1994). Similarly to the NMDA receptors, the regulation of perinatal kainate receptors by polyamines contributes to synapse formation and plasticity.

1.3. Polyamines and the cell cycle/ cell proliferation

The induction of proliferation has been considered one of the most important function of polyamines and the enzymes responsible for their synthesis, i.e. ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (adomet-DC). The concentration of free polyamines is low in non-proliferating cells, but a significant rise in polyamine levels is noted in cells entering the proliferation cycle. The amount of polyamines and the activities of polyamine-synthesizing enzymes increase rapidly in late G1 and in the transition of G2 to mitosis (Fredlund JO et al. 1995). In the S-phase and mitosis, polyamine catabolism by spermidine/spermine N^1-acetyltransferase (SSAT) overdrives the synthesis rate (Bettuzzi S et al. 1999). The increase as well as the following decrease in polyamine concentration are necessary for the proliferation cycle to progress accurately. The fluctuations of polyamines during the proliferation cycle are associated to sequential activation of cyclins and cyclin dependent kinases, especially cyclinE/cdk2 and cyclin A/cdk2 complexes (Gilmour SK, et al. 1999). One of the early signals in mitogenesis is the stimulation of ras, which further activates the mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) pathways (reviewed in McCubrey JA et al. 2007). MAPK and PI3K signalling stimulate the transcription and the activation of ODC (Wei LH et al. 2008, Origanti S et al. 2007, Shantz LM 2004, Flamigni F et al. 1997) via the transcription factor c-myc that binds to specific responsive elements in the promoter of ODC (Bello-Fernandez C et al. 1993). The resulting increases in ODC activity and polyamine concentration enhance the growth stimulus by reinforcing the expression of transcription factors, including c-myc, c-fos, and c-jun, creating a loop of positive feedback. Inhibition of ODC activity by DFMO leads to polyamine depletion and cell cycle block in G1/G0 by imbalancing the expression of the above-mentioned transcription factors and cell cycle inhibitors p21$^{Waf1/Cip1}$, p27^{Kip1}, p53, junD/AP-1, and TGF-β (Wang JY et al. 1993, Ray RM et al. 1999, Ravanko K et al.

2000, Patel AR et al. 1999, Patel AR et al. 1998). The fluctuation of polyamines during the proliferation cycle is associated with sequential activation of cyclins and cyclin-dependent kinases, especially cyclinE/cdk2 and cyclin A/cdk2 complexes (Gilmour SK et al. 1999).

1.4. In reproductive organs

ODC is highly expressed in the human reproductive organs, i.e. testis, ovary, and prostate. Polyamines were originally described in the semen, where they are secreted from the prostate. ODC and polyamines are required for spermatogenesis and proliferation of the germinal epithelium in murine testicular tissue (Alcivar AA et al. 1989, Qian ZU et al. 1985, Weiner KX et al. 1992). Transgenic mice overexpressing ODC developed infertility, with reduced size of testes and impaired spermatogenesis due to excessive amounts of putrescine (Halmekytö M et al. 1991, Hakovirta H et al. 1993). Ivanov et al. and Tosaka et al. (Ivanov IP et al. 2000, Tosaka Y et al. 2000) originally identified and cloned a testis-specific antizyme (AZ3 or OAZ-t) which is expressed exclusively in haploid germ cells, pointing to the need to tightly control the activity of ODC and of polyamines.

ODC has also regulatory functions in the gonads, unrelated to proliferation. Testosterone-producing Leydig cells of mature murine testis display a high ODC activity – even higher than that found in proliferating spermatogenic cells (Qian ZU et al. 1985). In Leydig and Sertoli cells, the activity of ODC is regulated by AZ1 (Tosaka Y et al. 2000). In steroid-hormone-producing cells of testis and ovary, the activity of ODC is stimulated by luteinizing hormone (LH) (Osterman J et al. 1983, Maudsley DV et al. 1974, Levine JH et al. 1973). Treatment with DFMO reduces the production of progesterone by inhibiting LH-induced preovulatory rise in ODC activity in mouse corpus luteum (Bastida CM et al. 2002). Intact ODC activity is also needed for normal folliculogenesis and luteinization in mouse ovary (Bastida CM et al. 2005). The gonadotropin-induced activation of ODC in the gonads suggests that ODC has a role in the regulation of steroid hormone synthesis or secretion.

2. Regulation of ODC

2.1. Antizymes

Antizymes (AZ) are proteins which sequester monomeric ODC molecules. AZs prevent the dimerization and formation of enzymatically active ODC (Heller JS et al. 1976, Murakami Y et al. 1994). Binding of AZ leads to conformational changes in ODC and exposure of the C-terminal PEST sequence (Li X et al. 1993) that provokes translocation to the 26S proteasome for degradation without ubiquitination (Murakami Y et al. 1992). AZ itself is not degraded together with ODC, but is recycled back to the cytoplasm. Due to antizyme-induced degradation, the half-life of ODC, only 10–20 min, is among the shortest known for proteins in mammalian cells (Murakami Y et al. 1985). A significant amount of ODC is in complex with AZ in mouse brain. ODC can be released and activated from this complex which might thus serve as a reservoir of rapidly activated enzyme (Laitinen PH et al. 1986).

Three isoforms of AZ have been recognized: AZ1 and 2 are distributed ubiquitously. The expression of AZ3, instead, is restricted to post-meiotic testicular germ cells (Ivanov IP et al. 2000, Tosaka Y et al. 2000) in which it is assumed to prevent the accumulation of cytotoxic amounts of polyamines. The recent results suggest a role for AZ3 also in the formation of head and tail connection in sperm (Tokuhiro K, et al. 2009). All three antizymes induce inhibition and degradation of ODC, although the expression level of AZ2 is lower (Ivanov IP et al. 1998, Zhu C et al. 1999, Snapir Z et al. 2008b). AZ1 and 2 also interact with yet unrecognized polyamine transporters and block the uptake of polyamines when the intracellular concentration is increasing (Suzuki T et al. 1994, Mitchell JL et al. 1994).

An increased polyamine content protects AZ from ubiquitin-mediated degradation and enhances AZ expression by affecting the rate of ribosomal frame-shifting (Matsufuji S et al. 1995, Palanimurugan R et al. 2004). AZ1 contains also a mitochondrial targeting motif. It is transported to the mitochondrial membrane where it depolarizes the membrane and activates the caspase cascade leading to the induction of apoptosis (Gandre S et al. 2003, Liu GY et al. 2006).

Over-expression of AZ1 inhibits cell proliferation and growth via ODC inhibition and reduction of the polyamine content (Murakami Y et al. 1994). Furthermore, in prostate cancer cells, failure of AZ induction is related to unregulated proliferation (Koike C et al. 1999). Expression of AZ in transgenic mouse reduced tumorigenesis in the skin (Feith DJ et al. 2001). Thus, AZ can be considered a tumor suppressor. Depletion of AZ in cultured cells leads to over-duplication of centrosomes, whereas the silencing of antizyme inhibitor (AZIN) reduces centrosome abnormalities (Mangold U et al. 2007). These data suggest that AZ and AZIN are connected to the early stages of carcinogenesis in which the loss of tumor suppressors triggers defects in centrosome functioning.

Recent data suggest that antizyme is not solely a regulator of ODC but also controls the degradation of other proteins that participate in growth regulation, e.g. cyclin D1, Smad1, and Aurora-A (Newman RM et al. 2004, Fong LY et al. 2003, Gruendler C et al. 2001, Lim SK et al. 2007)
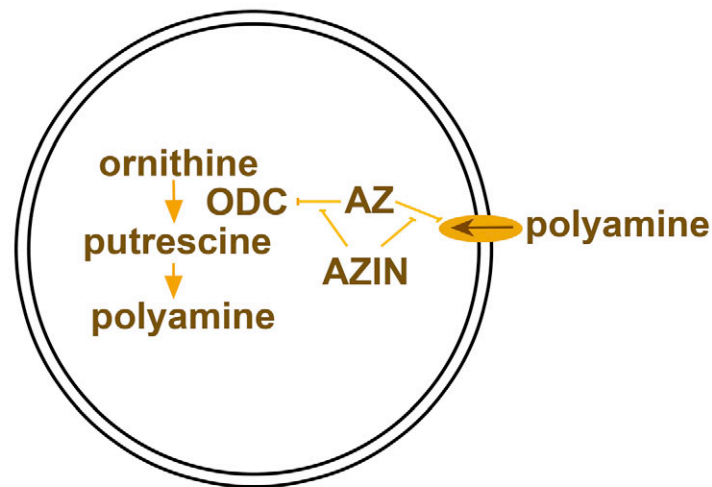

Figure 2. *The interplay of antizyme (AZ), antizyme inhibitor (AZIN), and ornithine decarboxylase (ODC) in the regulation of polyamine concentration. ODC catalyzes the synthesis of putrescine which is a precursor for higher polyamines, spermidine and spermine. AZ inhibits the activity of ODC and blocks the uptake of polyamines when the concentration of polyamines increases. AZIN counteracts AZ which leads to an increased polyamine content.*

2.2. Antizyme inhibitors

Antizyme inhibitors (AZIN) have arisen from *ODC* by gene duplication (Kidron H, et al. 2007) and thus share a high degree of sequence similarity with ODC (Murakami Y et al. 1996). Due to the homology, AZINs bind AZs, with even higher affinity than ODC, and thus liberate ODC from the heterodimer complex with AZ resulting in the formation of active homodimers and increased ODC activity (Mangold U et al. 2004, Fujita K et al. 1982). The binding of AZIN to AZ blocks also the inhibition of polyamine transporters mediated by AZ, and the uptake of polyamines is enhanced (Keren-Paz A et al. 2006, Snapir Z et al. 2008a). The binding of AZ to AZIN or ODC is reversible, and the equilibrium is constantly monitored and adjusted by the concentration of polyamines. Even though AZ promotes ODC for degradation, it actually protects AZIN from ubiquitination and targeting to proteasomes (Bercovich Z et al. 2004). In biochemical assays, AZIN binds all known AZs, AZ1-3 (Mangold U et al. 2004). AZIN remains a monomer under physiological conditions and it is unable to bind the cofactor, pyridoxal-L-phosphate, which is needed for the enzymatic activity of ODC (Albeck S et al. 2008).

AZIN increases the concentration of polyamines and has been demonstrated to promote proliferation and even transformation, whereas siRNA mediated down-regulation of AZIN expression reduces proliferation (Keren-Paz A et al. 2006, Kim SW et al. 2006, Choi KS et al. 2005). RNA interference of AZIN causes also the accumulation of multinucleated cells (Murakami Y et al. 2009). Interestingly, the same phenomenon was detected in cells in which the expression of ODC was constitutively targeted to the membrane (Heiskala M et al. 1999). In addition, the transcription of AZIN is increased by growth stimuli, prior to induction of ODC (Nilsson J et al. 2000). During the cell cycle, AZIN is activated similarly to ODC in late G1 and again in G2/M, and during mitosis (M) it is located in the centrosome analogously to AZ (Murakami Y et al. 2009). The growth-promoting activity of AZIN may not be solely dependent on the neutralization of AZ, since AZIN has been demonstrated to stabilize cyclin D1 independently of the AZ-binding (Kim SW et al. 2006). By using the gene-trap technique, a mouse line with a disrupted *AZIN* was generated (Tang H et al. 2009). The deletion turned out to be lethal at P0 and these mice had diminished levels of ODC activity, putrescine, and spermidine indicating

their importance for AZIN in embryogenesis. This experiment also demonstrated the *in vivo* functional role of AZIN in polyamine regulation. The expression of AZIN1 is elevated in human gastric cancer compared to normal gastric tissue (Jung MH et al. 2000) as well as in ras-transformed fibroblasts (Keren-Paz A et al. 2006). AZIN also promotes the survival of various types of cancer cells via activation of ODC under hypoxic conditions (Svensson KJ et al. 2008). In carcinogenesis, activation of ODC is considered to be an early step in malignant transformation. It has, however, been postulated that the activation of ODC might actually proceed via induction by AZIN (Keren-Paz A et al. 2006). ODC activation itself leads to a rapid increase in the amount of AZ, leading to the reciprocal diminution of ODC activity and polyamine uptake. In contrast, the activation of AZIN blocks AZ and promotes cell growth via sustained polyamine accumulation.

2.3. Spatial regulation of ODC

Intracellular compartmentalization of polyamines is functionally of great importance, since polyamines participate simultaneously in various cellular functions in the nucleus, mitochondria, plasma membrane, secretory vesicles, and cytoplasm. Investigations on the localization of ODC have proven difficult due to its extremely short half-life, and thus minute amounst of detectable protein. On the other hand, problems have been encountered in determining the compartmentalization of polyamines with the larger pool of bound polyamines compared to freely recruitable ones.

Polyamines take part in the modulation of various cellular signalling cascades for which they are either synthesized *de novo* or transported from extracellular spaces. Polyamines bound to nucleic acids and proteins are considered rather inactive in the event of the rapid recruitment for signalling (Watanabe S et al. 1991). Mitogenic signalling translocates ODC to the nucleus (Schipper RG et al. 2004, Schipper RG et al. 1999), possibly in connection with antizyme that is considered to regulate the nucleocytoplasmic shuttling of ODC. Indeed, AZ contains two nuclear export signals (Murai N et al. 2003). Immunochemical stainings from different cell lines indicate that AZ is mainly localized to the nucleus in actively proliferating cells, whereas in rat

brain it is also found in the cytoplasm (Mangold U et al. 2007, Schipper RG et al. 2004, Kilpelainen P et al. 2000). However, the nuclear transport of AZ is deemed important during the development of the rat central nervous system (Gritli-Linde A et al. 2001). Epitope-tagged AZIN1 has also been detected in the nucleus of proliferating, cultured cells (Lopez-Contreras AJ et al. 2009) suggesting that reciprocal activities of AZ and AZIN1 mediate the fluctuations in ODC activity during the cell cycle. However, the role of ODC in the synthesis of nuclear polyamines needs to be investigated further, since the presence of other enzymes needed for polyamine synthesis have not been described for the nucleus. If the function of nuclear proteasomes is restricted, ODC accumulates in the nucleus, indicating that ODC degradation targeted by AZ occurs also in the nucleus (Gritli-Linde A et al. 2001). AZ might have a more potent role in the degradational targeting in the nucleus, since AZ mediates also the degradation of the oncogene Aurora-A, the function of which is related to the progression of mitosis (Lim SK et al. 2007). During transition from prophase to telophase in mitosis, the proportions of AZ and AZIN1 are located in the centrosomes where they facilitate the completion of mitosis (Murakami Y et al. 2009). Overactivity of AZ leads to a decrease in the number of centrosomes, whereas the increased activity of AZIN1 is followed by an accumulation of excess centrioles (Mangold U et al. 2007). After mitosis, the entire orchestra of polyamine regulators, ODC, AZ1, AZIN1, and AZIN2, is detected in the perinuclear space (Mangold U et al. 2007, Schipper RG et al. 2004, our unpublished data). In addition to the centrosomes, which are located perinuclearly, ODC has been identified in the rough endoplasmic reticulum of human neurons (Bernstein HG et al. 1999). Perinuclearly, phosphorylated ODC colocalizes also with the keratin meshwork, the disruption of which is accompanied by a diffuse cytoplasmic spread of ODC (Pomidor MM et al. 1999). Specific phosphorylation of serine 167 in the p47^{PHOX}-like membrane-translocation motif relocates ODC to the plasma membrane upon cell activation (Heiskala M et al. 1999).

The induction of apoptosis is accompanied by an increase in ODC activity, and the resulting accumulation of putrescine is assumed to contribute to the activation of apoptotic signalling cascades. However, polyamines have also been proposed to play an antiapoptotic role (for a review, see Schipper RG et al. 2000). Conversely, the inhibition of ODC by DFMO and the depletion of polyamines protect cells from

apoptosis initiated by both extrinsic (receptor-induced) and intrinsic (mitochondria-derived) pathways (Ray RM et al. 2000). Although ODC itself has not been detected in mitochondria, AZ1 contains an N-terminal motif for mitochondrial targeting (Gandre S et al. 2003). An overexpression of AZ1 in hematopoietic cells leads to its accumulation in mitochondria, which is subsequently followed by caspase cascade- and cytochrome c-mediated apoptosis (Liu GY et al. 2006). Apoptosis, in which partly overlapping signalling cascades with growth induction are activated, exemplifies the necessity of compartmentalization and localized regulation of polyamines and the regulators of their synthesis.

In order to function as neuromodulators in neuronal ion channels, the concentration of polyamines needs to be regulated rapidly: both the uptake and release of polyamines needs to be controlled upon signalling. Neocortical rat glia cells are capable of efficiently transporting polyamines from the extracellular space and storing them intracellularly. Polyamines are also recruited to synaptic vesicles and synaptosomes in neurons (Masuko T et al. 2003). Conversely, spermine is released from hippocampal slices to the extracellular space upon depolarization. Polyamine transporters, still uncharacterized at the molecular level, are found in most cell types. These transporters accept a wide range of structural analogs of polyamines, and the net influx through them is enhanced by the depletion of polyamines (Cullis PM et al. 1999). Instead, if the intracellular concentration of polyamines is increasing, the transporters are inhibited by AZ (Suzuki T et al. 1994, Mitchell JL et al. 1994). Cells contain vast amounts of polyamine-sequestering vesicles (PSV) into which the uptaken polyamines are believed to be accommodated by H+:polyamine carriers (Cullis PM et al. 1999, Soulet D et al. 2004). Free polyamines apparently do not float in the cytoplasm, but are rather stored in vesicles so as to be rapidly released upon stimulus. It has been postulated that PSVs may serve as a major reservoir of polyamines, a mechanism which prevents the cytotoxic effects of free polyamines and provides a readily available tool for signalling purposes (Poulin R et al. 2006). In accordance with this model, the application of NMDA in order to activate its receptor has been demonstrated to induce rapid release of spermidine and spermine to the extracellular space (Fage D et al. 1992). In addition to their regulators, polyamines have also been described to relocate intracellularly upon signalling. Proliferating and

migrating cells of the retinal pigment epithelium recruit polyamines from membrane patches to cytoplasmic granules (Johnson DA et al. 2002).

3. The actin cytoskeleton

3.1. The regulation and functions of Rho

A cell is a three-dimensional structure in a three-dimensional environment. It is part of a larger complex of other cells, and its viability and functions are mainly determined by the changes in its near surroundings. When the environment creates tension to the cell boundaries leading to changes in the cell shape or adhesions to the environment, signal cascades are activated inside the cell leading to appropriate actions to respond to these changes. Important mediators between mechanical rearrangements and chemical cascades are cytoskeleton inside the cell and adhesion points to the surrounding cells and extracellular matrix in the plasma membrane{{51 Ingber,D. 1999; }}. Small G-proteins, Rhos, play an essential role in the regulation of both adhesions and cytoskeletal rearrangements {{47 Ridley,A.J. 1992; }}. Rho (referring to RhoA, B, and C) belongs to a family of Rho GTPases comprised of 26 known members of which the most studied are RhoA, Rac1, and Cdc42, and to a larger superfamily of ras-proteins (Takai Y et al. 2001). Numerous members of the Rho family proteins participate in various cellular processes including proliferation, migration, cell polarity, and vesicle trafficking (Jaffe AB et al. 2005). Rho functions as a main regulator of actin polymerization. Activation of Rho in cultured cells leads to the formation of actin stress fibers and focal adhesions (Ridley AJ et al. 1992). Rho is active in its GTP-bound form, whereas hydrolysis of GTP to GDP renders it inactive and unable to interact with its downstream effectors. The activity of Rho is regulated by various proteins belonging to three groups (Rossman KL et al. 2005, Kaibuchi K et al. 1999). The GTPase-activating proteins (GAP) catalyze the intrinsic hydrolysis of GTP to GDP, leading to inactivation of the Rho proteins. The guanine nucleotide-exchange factors (GEF) activate Rho proteins by stimulating the change of GDP to GTP. GEFs selectively recruit proteins of the Rho family to the site of action where they form multimolecular complexes with appropriate downstream effectors. Nearly 70 different GEFs have been described with pleiotropic functions in the pathogenesis of cancer, invasion of microbes, and development (Rossman KL et al.

2005). The guanine nucleotide-dissociation inhibitors (GDI) bind GDP-Rho and keep it in an inactive form in the cytoplasm. Rho GAPs, GEFs, and GDIs regulate the activity and localization of Rho, and the availability of selective effectors.

The Rho proteins are post-translationally modified by attachment of an isoprenyl group to the CAAX box in the C terminus (Hori Y et al. 1991). The isoprenyl group targets the protein to specific subcellular membranes (Hori Y et al. 1991). In the case of RhoA, the geranyl-geranyl tail attaches it to phospholipids in the plasma membrane. GDI-binding to Rho-GDP induces a change of conformation in such a way that the isoprenyl tail of Rho is hidden (DerMardirossian C et al. 2005). This prevents the membrane translocation of Rho and its interactions with downstream effectors. Rho can also be post-translationally phosphorylated. Several bacterial toxins modify Rho by ADP ribosylation, glycosylation and transamidation. Transamidation, consisting of deamidation and polyamination, is catalyzed by bacterial cytotoxic necrotizing factors with transglutaminase activity (Schmidt G et al. 2001). This leads to constitutive activation of Rho by preventing the hydrolysis of GTP (Masuda M et al. 2000). Mammalian transglutaminases also transamidate Rho, although its functional significance is still unknown (Flatau G et al. 1997).

Actin nucleators, protein kinases, and phospholipases are downstream targets of the Rho family proteins. Rho itself interacts with and regulates many functionally diverse downstream effectors which initiate a network of signalling cascades involved in transcription and cytoskeletal reorganization (Bustelo XR et al. 2007). Rho GEFs tether the effectors to the plasma membrane where Rho is located and directs downstream signalling (Buchsbaum RJ 2007). The interaction of Rho with effectors induces conformational change in downstream targets; this is often needed for their activation and assembly to hot spots (Bishop AL et al. 2000). The main effectors of Rho are ROCK (Rho-associated coiled-coil kinase) and mDia (mammalian homolog of Drosophila diaphanous). ROCK is a serine/threonine kinase that induces phosphorylation of the myosin light chain (MLC) phosphatase. This induces cross-linking of myosin II to actin and leads to the formation of contractile actomyosin filaments (Riento K et al. 2003). ROCK inactivates actin severing cofilin and stabilizes actin filaments via phosphorylation of the LIM kinase (Ohashi K et al. 2000). Rho induces polymerization of actin and the formation of actin fibers through

diaphanous-related formin, mDia (Li F et al. 2003). ROCK and mDia modulate the activities of each other, and their balance determines the type of stress fibers and the shape of a cell (Watanabe N et al. 1999, Tsuji T et al. 2002). Another effector, the citron kinase, regulates also the phosphorylation of MLC, but this interaction takes place in the cleavage furrow during cytokinesis (Madaule P et al. 1998). ROCK activity regulates microtubule collapse and the stabilization of different target proteins (Arimura N et al. 2000, Palazzo AF et al. 2001). By regulating the serum response factor and interacting with MEKK1, Rho promotes the transcription of genes encoding cytoskeletal components. Rho also contributes to the transcription of many growth-related genes (Miralles F et al. 2003, Gallagher ED et al. 2004).

The inhibition of Rho family proteins blocks the cell cycle at G1 (Olson MF et al. 1995). The influence of Rho on this block is regarded to result from the regulation of cyclin D1 and the cdk inhibitors, p21^{cip1} and p27^{kip1} (Welsh CF et al. 2001, Olson MF et al. 1998, Weber JD et al. 1997). The Rho target ROCK is involved in this regulation (Roovers K et al. 2003). Perhaps an even more important role for Rho in the regulation of the cell cycle takes place during mitosis. Mediated by ROCK and myosin II, Rho is needed for the correct positioning of centrosomes during prophase (Rosenblatt J et al. 2004). During cytokinesis, Rho also contributes to the formation and functioning of the contractile ring and to the localization of the cleavage furrow with its effectors anillin, citron kinase, mDia, and ROCK (Nishimura Y et al. 2006, Piekny AJ et al. 2008, Glotzer M. 2001). Rho regulates the *de novo* actin polymerization and the dynamics of the actomyosin complex in the contractile ring.

Actin is polymerized and its filaments are elongated at the leading front of the migrating cell, and depolymerized at the rear end focal contacts. The contractions of the actomyosin filaments move a migrating cell forward. At the rear end, Rho activates ROCK, leading to phosphorylation of the myosin light chain, contraction of the actomyosin filaments, and retraction of the rear of the cell (Riento K et al. 2003). ROCK is postulated to act also at the lateral sides of the cell in preventing inappropriate lateral protrusions (Worthylake RA et al. 2003). At the front of the cell, where excessive formation of contractile actin fibers needs to be restricted, the activity of Rho is controlled by Smurf1-dependent ubiquitination (Wang HR et al. 2003). Recent studies have, however, revealed that local activation of RhoA takes

place also at the edge of the protrusions and not only at the rear end of randomly migrating cells (Pertz O et al. 2006, Machacek M et al, 2009). The activity of RhoA in migrating cells is regulated by the localized activity of rhoGAPs, e.g. p190rhoGAP and DLC-1 (Kim TY et al. 2009, Bartolome RA et al. 2008). Furthermore, the relocalization of RhoA in migrating cells seems to be dependent on extracellular cues and the type of migration (Pertz O et al. 2006). Rho activates mDia1 in the front edge, leading to microtubule stabilization and alignment needed for migration. Rho-dependent activation of mDia at the rear end recruits c-src to focal adhesions, resulting in rac activation and turnover of the focal contacts (Yamana N et al. 2006). Intracellular relocalization of Rho balances the contractility of actomyosin fibers and enables the directional movement of a cell, for instance during organogenesis and chemotaxis.

Focal adhesions are macromolecular assemblies that attach cells to the extracellular matrix. The activation of Rho induces the assembly of focal adhesions by aggregation of vinculin and talin. The blocking of Rho activity, on the contrary, inhibits the assembly of actin stress fibers and focal adhesions (Ridley AJ et al. 1992). Detached and rounded cells, however, display high Rho activity which has been suggested to contribute to a more rigid actin cortex (Maddox AS et al. 2003). Rho participates also in the function of adherens junctions, which are involved in cell-cell contacts and in cytoskeletal dynamics. They convey extracellular signalling inside the cell and mediate mechanical forces. Rho proteins are postulated to function in the activity zones of adherens junctions where their activation and inactivation is balanced (Bement WM, et al. 2006, Yamada S, et al. 2007). Cadherins bind adjacent cells to each other in adherens junctions, and intracellular catenins link cadherins to the cytoskeleton (Hartsock A et al. 2008). The Rho-mediated formation and maintenance of adherens junctions is tightly regulated. Mechanical forces and signal transduction pathways of attachments activate Rho. Selective inactivation of Rho is mediated by rac1-recruited p120-catenin and p190rhoGAP (Wildenberg GA et al. 2006), and an adhesion-dependent negative feedback-loop maintains the balance of the Rho activity (Ren XD et al. 1999). p120-catenin binds Rho-GDP in adherens junctions and sequesters it from activation by GEFs; this leads to the local regulation of actin reorganization (Noren NK et al. 2000).

Rho GTPases are also involved in tumor initiation, progression and metastasis (Narumiya S et al. 2009). Rho proteins are usually not mutated in cancers, but their overactivity is found in a large variety of human tumors (Sahai E et al. 2002a). The elevated activity of Rho proteins in cancer cells might explain partly the increased motility of neoplastic cells. Enhanced expression of RhoC is screened from highly metastatic melanoma cells by DNA arrays. Furhermore, dominant-negative Rho inhibits metastasizing (Clark EA et al. 20009). Experiments performed with mutant Rho proteins in cultured cells suggest that they play a role in tumor initiation. Constitutively active forms of RhoA and Rac1 are able to induce cell transformation, whereas dominant-negative mutants of these proteins block ras-induced transformation (Qiu RG et al. 1995a, Qiu RG et al. 1995b, Prendergast GC et al. 1995). The activity of Rho is high in ras-transformed cells, and it is considered a prerequisite for ras-induced transformation (Qiu RG et al. 1995b). ERK-MAP kinases activate Rho and also regulate the downstream signalling of Rho, thus promoting pathways of transformation (Sahai E et al. 2001). Increased proliferation occurs in tumor initiation. In addition to its inhibitory effect on inhibitors of cyclin-dependent kinases, Rho influences proliferation and the cell cycle by regulating ROCK and MEK/ERK. Active Rho enhances the activity of transcription factors (e.g. stat3, NF-κB, serum response factor) (Benitah SA et al. 2004, Montaner S et al. 1998, Benitah SA et al. 2003, Psichari E et al. 2002).

Rho and ROCK activity is connected especially to a bleb-associated/ameboid mode of motility in which cells respond to chemo-attractants by relocating ezrin in a Rho-dependent fashion, in the direction of movement. The elongated-cell-type of motility is less dependent on the activity of Rho/ROCK (Sahai E et al. 2003). In src-transformed cells, which migrate in an elongated fashion, Rho is relocalized to podosomes. Rho may be needed for the formation of podosome structures implicated in tumor cell invasion (Berdeaux RL et al. 2004). The src-oncogene regulates the local activation and the cytosol-membrane cycling of Rho by inactivating the phosphorylation of Rho GDI (DerMardirossian C et al. 2006). Smurf1, a ubiquitin ligase, induces degradation of Rho in the periphery of tumor cells and thereby regulates the formation of protrusions and the motility of the cells (Wang HR et al. 2003, Sahai E, et al. 2007). In colorectal carcinoma cells, a low activity of Rho favors the maintenance of adherens junctions via mDia, whereas an increased activity of Rho

and ROCK promotes the collapse of junctions and enhances migration (Sahai E et al. 2002b). The inhibition of RhoA by RNA interference or dominant-negative mutants has been found to attenuate the proliferation and tumorigenicity of gastric cancer cells and to enhance their sensitivity to chemotherapy (Liu N et al. 2004). A chemotherapeutic effect has also been achieved with a specific ROCK inhibitor, Y-27632, which prevented the establishment of transplanted tumors in mice (Itoh K et al. 1999). The same inhibitor also abolished the metastasizing capacity of invasive prostate cancer cells in mice (Somlyo AV et al. 2000).

3.2. The effect of polyamine regulation on the cytoskeleton

Cells kept in polyamine-free culture and depleted of polyamines by inhibiting ODC activity lack normal actin fibers, thick stress fibers and microtubules (Pohjanpelto P et al. 1981, McCormack SA et al. 1994). Such cells may appear rounded with a considerably increased actin cortex and reduced lamellipodia, or elongated with a single thick actin bundle. The addition of polyamines restores the cytoskeleton and cell shape to normal. Inhibition of ODC not only distorts actin stress fibers, but also affects rER which perturbs the synthesis of proteins (Parkkinen JJ et al. 1997). Small multivalent cations, including polyamines, induce the bundling of actin fibers (Sowa GZ et al. 2006) and also enhance the nucleation and elongation of microtubuli by facilitating the diffusion of tubulin (Mechulam A et al. 2009). In rapidly dividing cells, phosphatidylinositol 4,5-bisphosphate mediates the effect of polyamine on actin nucleation (Coburn RF et al. 2006). An altered distribution of tropomyosin, a stabilizer of actin fibers, is found in polyamine-depleted cells (McCormack SA et al. 1994). Furthermore, depletion of polyamines lowers the amount and activity of Rho and its downstream effector ROCK (Rao JN et al. 2003). Polyamines thus influence the dynamics of the cytoskeleton in various ways. In ras- and RhoA-transformed cells, the morphological changes are reversed by the inhibition of ODC activity (Shantz LM et al. 1998). A similar effect was detected in temperature-sensitive src-transfected cells cultured in polyamine-depleted medium. Inhibition of ODC by treatment with DFMO blocked the src-induced depolymerization of filamentous actin and morphological transformation (Höltta E et al. 1993).

Cells treated with DFMO also display inadequate attachment to the extracellular matrix and are incapable of spreading. Polyamine-depleted cells contain reduced amounts of focal adhesion kinase (FAK), and the phosphorylation of FAK and paxillin is inhibited (Ray RM et al. 2001). This leads to a failure to form protein complexes at the adhesion sites and prevents their binding to the actin stress fibers. Furthermore, polyamines are needed for integrin assembly and signalling in the adhesions.

Johnson et al. have studied the role of polyamines in intestinal healing, in which the migration of enterocytes is a pivotal event. Wound healing and migration of intestinal epithelial cells (IEC-6) were inhibited by treatment with DFMO and the subsequent depletion of polyamines (Banan A et al. 1996, Ray RM et al. 2002). Exogenous polyamines were nevertheless able to restore the mobility of IEC-6 cells. Defects in the dynamics of the actin and microtubule cytoskeleton caused the migratory inhibition. Polyamine depletion reduced $[Ca^{2+}]$ mediated by voltage gated K^+ channels leading to the inhibition of RhoA, ROCK, and phosphorylation of the myosin light chain (Rao JN et al. 2003). Polyamines participate in the regulation of the cytoskeleton in normal proliferation and migration, as well as in malignant transformation. Polyamines are apparently involved in various signalling cascades in which Rho plays an important role.

4. Polyamines and ODC in diseases

4.1. Cancer

If ODC is over-expressed in NIH 3T3 murine fibroblasts, the cells undergo transformation and show anchorage-independent growth in soft agar (Auvinen M et al. 1992, Moshier JA et al. 1993). Inoculation of such ODC-overexpressing cells in nude mouse gave rise to highly-vascularized fibrosarcomas capable of invasive growth (Auvinen M et al. 1997). ODC is surmised to function at the cross-roads of signal transduction pathways downstream of the oncogenes src, myc, and ras, including raf/ERK/MEK and PI3K pathways (Höltta E et al. 1993, Shantz LM 2004, Flamigni F et al. 1997, Auvinen M et al. 2003). In cells transformed by oncogenes or carcinogens, the activity of ODC remains constantly elevated (Hölttä E et al. 1993,

Hölttä E et al. 1988, Gilmour SK et al. 1986). The inhibition of ODC activity by DFMO, however, retained the normal morphology of src-transformed cells, thus emphasizing the role of ODC and polyamines in the transformation process (Höltta E et al. 1993). Experiments on transgenic mice have provided substantial evidence to support the participation of ODC and polyamines in tumorigenesis. The most studied model is skin tumorigenesis in these mice, in which ODC is expressed under the K6 keratin promoter. The over-expression of ODC in these mice promotes tumor formation after their treatment with carcinogens, UV-radiation, or ras-activation (Pegg AE et al. 2003, Hayes CS et al. 2006, George K et al. 2005). Double-transgenic mice over-expressing both *ODC* and *v-Ha-ras* developed spontaneous tumors (Smith MK et al. 1998). Oral administration of DFMO delayed the formation of skin tumors and regressed existing ones in transgenic mice over-expressing MEK1 under the keratin 14 promoter. This indicates that ODC activity is needed for the initiation and maintenance of tumors (Feith DJ et al. 2005). However, ODC-transgenic mice in which ODC is under its own promoter and over-expressed in all tissues are not prone to spontaneous tumorigenesis in life-long surveillance with the exception of skin papillomas after two-stage chemical induction (Alhonen L et al. 1995, Halmekyto M et al. 1992). Homozygous deletion of the ODC gene (knockout) is embryonally lethal. Haploinsufficient $ODC^{+/-}$ mice develop fewer tumors during their life-span as compared to their normal litter mates (Guo Y et al. 2005). Over-expression of the ODC inhibitor, antizyme, in the skin of transgenic animals prevents carcinogen-induced tumorigenesis (Feith DJ et al. 2001). Elevated levels of polyamines have been measured in human colon, breast, and prostate cancers (Leveque J et al. 2000, Mohan RR et al. 1999, Hixson LJ et al. 1993). Acetylated catabolic derivatives of polyamines in urine have been studied and used as a diagnostic marker for cancer (Inoue H et al. 2005). In addition, single-nucleotide polymorphism in ODC promoter is reported to predict a risk for colon polyps and cancer (Martinez ME et al. 2003).

The inhibitors of polyamine biosynthesis and polyamine analogs are the focus of study as potential chemotherapeutic agents. The ODC inhibitor, DFMO, lowers the concentration of putrescine and spermidine, and inhibits proliferation of malignant cells in cultures (Prakash NJ et al. 1980). DFMO can be administered orally or peritoneally, and it is non-toxic (Griffin CA et al. 1987, Abeloff MD et al. 1986). The efficacy of DFMO in clinical trials has, however, been disappointing. Polyamine

metabolism is regulated by various factors, and thus the inhibition of ODC has proved inadequate for achieving a significant decrease in the polyamine content of tumors *in vivo*. DFMO is now being studied in combination with non-steroidal anti-inflammatory drugs as a potential chemopreventive agent of epithelial cancers (Meyskens FL Jr. et al. 1999, Meyskens FL Jr et al. 2009). Polyamine analogs, various forms of which have been synthesized, have a multi-level impact on polyamine metabolism, and thus seem to be more potent chemotherapeutic agents. In order to function efficiently as a chemotherapeutic agent, a polyamine analog needs to influence polyamine synthesis, catabolism and uptake. It can also be presumed that part of the efficacy is explained by the binding of polyamine analogs to the same structures as the naturally occurring polyamines (for a review, see Casero RA Jr. et al. 2007). Polyamines and their analogs are also used as vehicles to provide non-viral routes inside a cell for other chemotherapeutic and anti-parasitic compounds (Holley JL et al. 1992, Delcros JG et al. 2006).

4.2. Brain pathologies

The induction of ODC activity results in increased concentrations of putrescine in the CNS. This is a general response to various physiological and pathological incidents, e.g. seizures, traumatic injuries, Alzheimer's disease, ischemia, as well as chemical, radiation, and electrical stimulation.

Experiments with mice and rats as well as studies on tissue cultures have shown that polyamines and ODC are connected to ischemic responses, especially during the reperfusion phase. Transient focal cerebral ischemia induces ODC activity, followed by accumulation of putrescine and depletion of the higher polyamines, spermidine and spermine (Paschen W et al. 1991). This effect is particularly pronounced in the penumbra on the ipsilateral side of the ischemic lesion, but also in apoptotic cells of the hippocampus after mid-cerebral artery occlusions (Baskaya MK et al. 1997, Sauer D et al. 1992, Muller M et al. 1991, Maeda M et al. 1998, Keinänen R et al. 1997). The same phenomenon has been detected also in ischemic heart (Zhao YJ et al. 2007, Zhao YJ et al. 2009). These findings have led to the assumption that ODC induction provokes the onset of ischemic damage and that putrescine mediates cytotoxic effects in neurons. However, experiments with transgenic animals that over-express ODC provide a different picture, pointing to a neuroprotective role of

polyamines and ODC. The infarct volume was smaller and the reperfusion damage developed more slowly than in the wild-type littermates or DFMO-treated animals (Lukkarinen JA et al. 1998, Lukkarinen JA et al. 1999). Long-lasting up-regulation of ODC expression was detected also in areas where no reperfusion lesions had developed. Intracranial infusion of putrescine has even been reported to provide protection against ischemia-induced neuron death (Keinänen R et al. 1997, Gilad GM et al. 1991). Results reinforcing the neuroprotective role of polyamines were obtained from experiments on rat brains subjected to continuous infusion of antisense oligonucleotide to ODC during transient mid-cerebral artery occlusion. Inhibition of ODC predisposed the animals to increased infarct volume, motor deficit, and mortality (Raghavendra Rao VL et al. 2001). Maternal hypoxia elevated the activity of ODC and the concentration of polyamines in the brain of mouse embryos. However, no neurotoxic effects were detected in the embryonal brain (Longo LD et al. 1993). Spermine, but not putrescine or spermidine, had a protective role in primary cultures of hippocampal and pyramidal neurons. It promoted the survival of neurons in a NMDA-dependent fashion and served as a scavenger of free radicals (Abe K et al. 1993, Ha HC et al. 1998). This indicates that different polyamines have specific functions. Furthermore, the effect of polyamines on the development of ischemic lesions is postulated to depend on the NMDAR activity: antagonists of NMDAR blocked the ischemia-induced activation of ODC, and inhibition of ODC prevented NMDAR-induced cytotoxicity (Keinänen R et al. 1997, Markwell MA et al. 1990). In conclusion, ODC and polyamines contribute to the modulation of ischemic brain trauma. There is still controversy, however, regarding the exact mechanisms, as well as the ultimate role that polyamines play in neuroprotection and cytotoxicity.

The expression and activity of ODC is locally increased in Alzheimer's disease (Bernstein HG et al. 1995, Morrison LD et al. 1998). Accumulation of spermidine has been found together with reduced levels of putrescine and S-adenosylmethionine, indicating enhanced synthesis of higher polyamines (Morrison LD et al. 1995, Morrison LD et al. 1996). Immunohistochemistry of diseased brain tissue has revealed accumulation of ODC in neocortical pyramidal cells and interneurons. Not only the amount of protein seemed to be elevated, but also the number of ODC-positive cells was increased (Bernstein HG et al. 1995, Nilsson T et al. 2006a). Furthermore, ODC was intracellularly redistributed. The normal nuclear and

perinuclear localization of ODC was spread more diffusely in the cytoplasm of neocortical, hippocampal, and Purkinje neurons in AD (Nilsson T et al. 2006a). ODC was detected also in the dendrites and axons of cortical pyramidal cells in AD brains, contrary to that of normal brain tissue (Bernstein HG et al. 1995).

Aberrant accumulation of β-amyloid into the extracellular space is considered important in the pathogenesis of AD. Activation of the β-amyloid precursor protein, which functions as a cell surface receptor, causes a rapid increase in the expression of ODC (Nilsson T et al. 2006b). β-amyloid accelerates the generation of reactive oxygen species (ROS), and this in turn induces ODC activity and the production of polyamines (Yatin SM et al. 1999). The uptake of polyamines is also upregulated by β-amyloid stimulation which, together with increased ODC activity suggests an underlying induction of AZIN. The generation of ROS is enhanced by the binding of β-amyloid oligomers to NMDARs, and is dependent on NMDAR activity (De Felice FG et al. 2007). Activation of NMDAR upregulates the expression of ODC in cultured neuronal cells (Nilsson T et al. 2006a). Simultaneously, β-amyloid disturbs NMDAR-mediated long-term potentiation in the hippocampus and interferes with down-stream signalling of NMDAR (Yamin G 2009). Polyamines regulate NMDAR activity in various ways, mostly by enhancing ligand binding (Araneda RC et al. 1999, Benveniste M et al. 1993). A positive feedback loop is formed between ODC and NMDAR, since NMDAR has been found to stimulate ODC activity leading to enhanced synthesis of polyamines, which in turn amplify NMDAR signalling. Further evidence supporting functional coupling between ODC and NMDAR was obtained from ODC transgenic mice with impaired performance in spatial learning and memory (Halonen T et al. 1993), i.e. functions regulated by NMDAR signalling. Whether polyamines are neuroprotective or neurotoxic in AD is still unsolved. Nevertheless, ODC transgenic mice which have high ODC activity and putrescine levels, do not develop more age-related neurodegenerative changes in neurons or brains than their wild-type littermates (Alhonen L et al. 1995). This suggests that ODC activation itself does not have deleterious effects, but is rather an adaptive response to noxious stimuli. The accumulation of spermine in AD might indicate an attempt to neutralize free radicals formed by the overactivity of β-amyloid (Yatin SM

et al. 2001). There is evidence of the involvement of polyamines in other neurodegenerative diseases, e.g. Parkinson's disease, in which spermine enhances the aggregation of α-synuclein (Grabenauer M et al. 2008).

The overall view of polyamines and ODC in brain pathology is in favor of neuroprotective responses to pathological events, despite numerous controversial findings. Therapeutic interventions using the polyamine system have not advanced, however, except for the use of memantine in AD. Overactivity of NMDAR leading to cytotoxicity is prevented by memantine which interferes with the polyamine binding to NMDAR (Bresink I et al. 1995).

AIMS OF THE STUDY

The aim of this study was to investigate the functional factors involved in the regulation of polyamines and ornithine decarboxylase, and to search for a mechanistic link between the polyamine system and the dynamics of the actin cytoskeleton. The specific aims were:

– to investigate the polyamination of a small G protein, RhoA and the role of transglutaminase in polyamination
– to evaluate the relevance of polyamination in proliferation and transformation
– to investigate the influence of ODC activity and localization on RhoA regulation
– to characterize a novel ODC homolog from human brain
– to investigate the tissue-specific expression of AZIN2
– to gather basic knowledge of AZIN2 in order to determine its functional role.

MATERIAL AND METHODS

The material and methods used in this thesis work are described in detail in the original publications. The table below summarizes the methods used with a referral to the respective publication.

Method	Publication
Antibody production	III, IV
Cell culture	I, II
Cell cycle synchronization	I
cDNA cloning	I, II
DNA histogram (flow cytometry)	I
DNA purification	I-IV
DNA sequencing	I - IV
Dot blot	II
Fractionation of cells	I
Immunohistochemistry	III, IV
Immunofluorescence stainings	I, IV
Immunoprecipitation	I
In vitro translation	II
In situ hybridization	III, IV
Metabolic labeling	I
Microscopy, confocal	I
Microscopy, epifluorescence	I, IV
Light microscopy	I, III-IV
ODC-assay	I, II
PCR	I, II,
RT-PCR	II, IV
Transfection	I - IV
Transformation assay	I
SDS-PAGE	I, II
Sequence alignment	I
Site-directed mutagenesis	I
Validation of antibody	III, IV
Western blotting / SDS-PAGE	I-IV

RESULTS AND DISCUSSION

1. ODC and the cytoskeleton

1.1. Transglutaminase mediates polyamination of RhoA

The physiological polyamination of RhoA was studied in murine fibroblast cell lines (NIH 3T3 and Rat1 tsRSVLA29), and in a cell line of Chinese hamster ovary in which the ODC-protein was mutated and catalytically inactive (C55.7, called CHO ODC- in these studies). The CHO ODC- cell line was further transfected with active ODC (CHO ODC+), and with ODC incapable of membrane translocation (CHO PHOX). All of the above-mentioned cell lines were metabolically labeled with ^{14}C-putrescine or ^{14}C-spermidine. Separated cell lysates were visualized with both an antibody to RhoA and by autofluorography. Western blotting revealed two closely spaced bands, but only the faster moving band incorporated labeled polyamines. Furthermore, from the same cell lysates, RhoA was immunoprecipitated, and after SDS-PAGE and autofluorography, binding of labeled polyamines to RhoA was detected (I, Fig 1A). The results are in accordance with those of Masuda and coworkers, who have earlier reported that polyaminated RhoA displays faster mobility on SDS-PAGE than does unmodified RhoA (Masuda M et al. 2000). It is noteworthy that the peptide antibody used in our studies detects not only RhoA, but also RhoC, the function of which is less characterized but considered to be similar to that of RhoA. Therefore the possibility that RhoC also might be polyaminated cannot be ruled out. Polyamination of RhoA has previously been detected *in vivo* only in the presence of exogenous transglutaminase (*Bordetella* dermonecrotizing toxin) which catalyses polyamination of RhoA by covalent cross-linking of putrescine and spermidine to Gln63 (Masuda M et al. 2000, Shin DM et al. 2008). This modification activates RhoA by inhibiting its intrinsic and extrinsic GTPase activity, and leaves the G protein in a constitutively active, GTP-bound configuration. Earlier, the polyamination of RhoA has been shown only with additive transglutaminases, whereas in the present studies the polyamination was detected in all cell lines tested without addition of exogenous transglutaminases. Differences in the relative amounts of polyaminated RhoA were, however detected between cell lines, which might explain the discrepancy in detecting physiological polyamination of RhoA. In

addition, the detection of the polyamination of RhoA proved to be inaccurate from frozen and thawed samples. Only fresh prepared samples were thus used in the studies.

To further study the role of transglutaminase activity for the polyamination of RhoA, Rat1 tsRSVLA29 and CHO ODC+ cells were treated with 0.2 mM monodansylcadaverine (MDC), an inhibitor of tissue transglutaminase 2 (TG2). Western blotting of lysates from MDC-treated cells revealed greatly reduced amounts and even an absence of fast-moving, polyaminated RhoA (I, Fig 1B). This indicated that the catalytic activity of transglutaminase was needed for the polyamination of RhoA and further confirmed that the faster moving band of RhoA was modified by polyamination. These results are in accordance with the findings of Singh et al. who have earlier shown that the transamidation of RhoA is prevented by inhibiting the activity of transglutaminase (Singh US et al. 2001). Recently, the inhibition of TG2 and the following reduction in the polyamination of RhoA have been shown to prevent also the interaction of RhoA with its downstream effector, ROCK (Shin DM et al. 2008).

Transamidation activity of TG is dependent on high $[Ca^{2+}]$, and such a concentration has been linked only to pathological states. However, the present results suggest the transamidation of RhoA by polyamines to take place in physiological states including proliferation. Most likely high concentration of Ca^{2+} is achieved by local regulation.

1.2. Polyamination of RhoA and the cell cycle

Changes in the relative amounts of polyaminated RhoA were investigated in lysates collected at various stages of the cell cycle from synchronized NIH 3T3 cells. Western blotting with polyclonal antibody against RhoA revealed changes in the proportion of intact versus polyaminated RhoA. Faster moving, polyaminated RhoA dominated in cells entering the G2/M phases when the incorporation of radio-labeled spermidine was also detected by SDS-PAGE and autofluorography (I, Fig 2B). In contrast, unmodified RhoA was abundant during G1 and S. The impact of RhoA polyamination on the progress of the cell cycle was also studied in Jurkat cells treated

with two inhibitors of TG2 (0.2 mM MDC or 0.2 mM cystamine) for three days prior to flow-cytometric analysis. Inhibition of TG2 induced a relative block between G2/M and G1, with an increase from 6% to 29% of the cell population (with MDC) in G2/M without accumulation in G1 (I, Fig 2E). Overexpression of RhoA by a transient transfection sensitized these cells to a relative cell cycle block induced by inhibitors of TG2. Similarly, a reduction in the amount of polyaminated RhoA, obtained by transfecting the cells with a polyamination-deficient mutant of RhoA, RhoA-Q63A, blocked the cell cycle. 70% of the cells accumulated in G2/M. Treatment with inhibitors of transglutaminase 2 had no further effect on the accumulation of cells expressing RhoA-Q63A in G2/M.
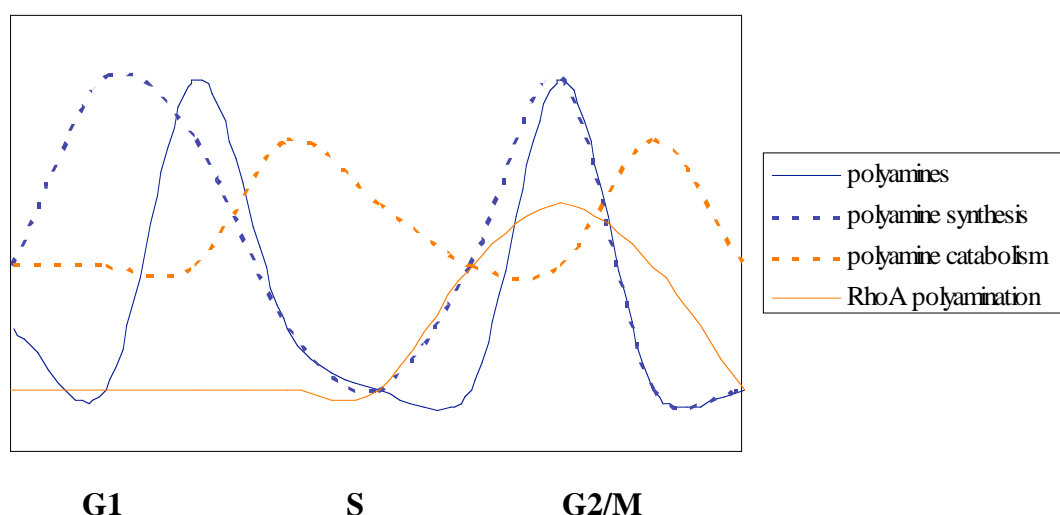

Figure 3. *The concentration of polyamines and the activities of their metabolizing enzymes fluctuate during the cell cycle. Polyamination of RhoA is detected simultaneously with the elevated amount of polyamines in G2/M phases.*

MDC and cystamine are amines that inhibit transglutaminase by competitive binding, although cystamine also directly inhibits transamidation. The activity of ODC and the relative amount of spermidine were measured from the cell lines used in these experiments in order to exclude the possibility that the detected cell cycle block was caused merely by the reduced polyamine synthesis induced by these compounds acting as polyamine analogs. No decrease in the activity or in the content of spermidine was detected in Jurkat or CHO ODC+ cells after treatment with MDC or cystamine (I, supplementary material). This proved that the effects of the compounds

were due solely to the inhibition of TG2. Since MDC decreased slightly the activity of ODC and also the content of spermidine in Rat1 tsRSVLA29 cells, only cystamine was used to treat this cell line.

These results show that the extent of polyamination of RhoA alters during the cell cycle, and that the fluctuation is required for proper progress of the cell division. In mitosis, RhoA affects centrosome positioning (Rosenblatt J et al. 2004) and is involved in creating contractile forces at the cleavage furrow during cytokinesis (Glotzer M 2001). It is conceivable that the activation of RhoA by polyamination during the G2/M phase is related to these functions.

1.3. Polyamination of RhoA and transformation

RhoA by itself is not a transforming protein, but it enhances migration and invasion of ras and src-transformed cells (Sahai E et al. 2003, Sahai E et al. 2001). To investigate the functional impact of the polyamination of RhoA on cell transformation, Rat-1 cells (Rat1 tsRSVLA29) were infected with a temperature-sensitive mutant of v-src (ts-v-src). Hölttä et al. have previously shown that the depletion of polyamines inhibits the ts-v-src-induced transformation at a permissive temperature, whereas the addition of exogenous polyamines restores the transformation (Höltta E et al. 1993). We compared the transforming capability of Rat1 tsRSVLA29 cells cultivated in the presence or absence of 0.25 mM cystamine. The control cells underwent morphological transformation at a permissive temperature, whereas the rate of transformation (I, Fig 4) and the polyamination of RhoA (I, Fig 1B) were repressed in cells treated with the TG2 inhibitor cystamine. These cells were sensitive for the over-expression of RhoA or RhoA-Q63A. Transiently transfected cells acquired a rounded morphology even at a non-permissive temperature and were detached more easily from the culture plate. Rounded cell morphology has previously been linked to the activity of RhoA and ROCK (Sahai E et al. 2003). Rounding of cells has also been detected in v-src transformed mouse fibroblasts transfected with constitutively active RhoA, RhoA-Q63L. The activity of RhoA (and RhoC) was spatially regulated in these cells and accumulated in the cortical ring of actin (Berdeaux RL et al. 2004). The present results are in accordance with these findings, and suggest that RhoA is an

important player in v-src-induced cell transformation, and that polyamination regulates the activity of RhoA.

1.4. ODC-mediated spatial regulation of RhoA and reorganization of actin

CHO ODC- cells and their derivatives CHO ODC+ and CHO PHOX were used for studying the impact of ODC on the spatial regulation of RhoA. Mutation of the p47^{PHOX} motif impairs the membrane translocation of ODC upon cell activation (Heiskala M et al. 1999). In addition, NIH 3T3 cells were transfected with an antisense oligonucleotide to AZ1 and Rat1 tsRSVLA29 cells with a polyamination-deficient mutant of RhoA (RhoA-Q63A). The reorganization of actin was investigated in these cells. The cell lines were cultivated in the absence of polyamines for three days and stimulated by hypo-osmotic stress to enhance the ODC activity (Poulin R et al. 1990) with the exception of Rat1 tsRSVLA29 cells.

Western blotting of total cell lysates revealed a robust polyamination of RhoA in CHO ODC+ cells (I, Fig 3A). The polyamination was less efficient in NIH 3T3 and Rat1 tsRSVLA29 cells, suggesting differences in the activity of TG2 between the different cell lines. In cells with normally inducible ODC activity and with ODC capable of membrane translocation, the reorganization of actin responded to changes in the activity of ODC. The induction of ODC by hypo-osmotic stress resulted in the formation of actin stress fibers which depolymerized when the activity of ODC declined (unpublished results from the present studies, Fig 4). Furthermore, no defects were observed in the progression of the cell cycle. Interestingly, NIH 3T3 cells with constitutively high ODC activity obtained by treatment with AZ1-antisense oligonucleotide were rounded, loosely attached, and lacked actin stress fibers (unpublished data).

The relative amount of polyaminated RhoA was lower in CHO PHOX cells, and especially in CHO ODC- cells compared to the CHO ODC+ cell line. The treatment of CHO ODC+ cells in polyamine-free medium with DFMO increased the amount of non-polyaminated RhoA (I, Fig 3B). This shows that catalytically active ODC and

ODC capable of membrane translocation are needed for the efficient polyamination of RhoA in the absence of external polyamines. Nevertheless, the polyamination of RhoA was not totally prevented by inhibition of the ODC-activity suggesting that the polyamination is not entirely dependent on ODC-activity. Ray et al. have shown that inhibition of ODC by DFMO decreases the activity of RhoA that is recovered by the addition of putrescine (Ray RM, et al. 2002). This is in accordance with present results of the roles of ODC and TG2 for the polyamination of RhoA.

The formation of actin stress fibers as a response to hypo-osmotic stress was repressed in CHO ODC- cells. In CHO PHOX cells, the reorganization of actin was delayed: stress fibers appeared later and resolved more slowly (unpublished results, Fig 4). The reorganization of actin was also disturbed in Rat1 tsRSVLA29 cells transfected with polyamination-deficient RhoA (RhoA-Q63A). These cells were rounded and loosely attached. The findings on stress fiber reorganization emphasize not only the importance of ODC-induction for the regulation of RhoA and actin reorganization, but also the demand for dynamic fluctuation in ODC activity.
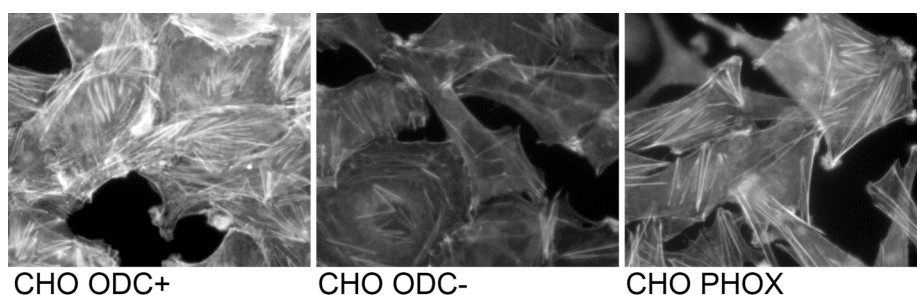

CHO ODC+   CHO ODC-   CHO PHOX

Figure 4. *The formation of actin stress fibers after 4 hours of hypo-osmotic shock. The cells were cultivated in polyamine-free media, and actin is visualized by rhodamine phalloidin staining.*

Analysis of the membrane and cytoplasmic fractions of CHO PHOX cells showed that the membrane fraction was devoid of ODC activity and, interestingly, also of both intact and polyaminated RhoA (I, Fig 3C). Only ubiquitinated forms of RhoA were found in the membranes of CHO PHOX cells. In CHO ODC+ cells both active

ODC and polyaminated RhoA were detected in the membrane fraction. However, if CHO ODC+ cells were treated with a synthetic peptide RLSVKFGA, which corresponds to the p47[PHOX] motif of ODC and prevents its membrane translocation (Heiskala M et al. 1999), both ODC activity and RhoA were absent from the membrane fraction (data not shown).

In CHO ODC+ cells cultured in the presence or absence of polyamines, confocal immunofluorescence microscopy with an antibody to RhoA revealed a concentration of RhoA close to the plasma membrane. In CHO PHOX cells, RhoA displayed a diffuse cytoplasmic distribution (I, Fig 3D). Some accumulation of RhoA was seen in the vicinity of the plasma membrane in CHO ODC- cells, but most of it remained dispersed over the cytoplasm. Analysis of CHO ODC+ cells transfected with RhoA-Q63A showed an accumulation of RhoA beneath the plasma membrane (I, Fig 3E). These findings demonstrate that an intact membrane translocation of catalytically active ODC is a prerequisite for the distribution of RhoA to the membrane actin cytoskeleton. It has been previously reported that inhibition of the ODC-activity decreased the amount of RhoA in the membrane fraction by 27% (Ray RM et al. 2002). However, the ODC-regulated spatial distribution of RhoA is independent of the polyamination of RhoA.

2. The structure and distribution of AZIN2

2.1. Comparison of the structures of ODC and AZIN2

As *AZIN*s are considered to have emerged via gene duplication of *ODC*, the homology between these proteins is high. The identity between AZIN2 (SV1; formerly named ODC-paralogue, ODC-p) and ODC is 54%, and the similarity is 75%. The corresponding values for AZIN2 and AZIN1 are 45% and 66%, respectively (II, Fig. 2).

ODC is fully active only as a homodimer, and it has two active sites at the interphase of the monomers. Using crystallographic and biochemical assays, Albeck et al. (2008) showed that AZIN1 appears as a monomer under physiological conditions). Recently, the amino acids crucial for the lack of dimerization of AZIN1 were determined to be

Ser 277, Ser 331, Glu 332, and Asp 389 (Su KL, et al. 2009). Even though no data yet exist on the nature of AZIN2 dimerization, the amino acids Ser 277 and Ser 331 are also dissimilar in AZIN2 compared to ODC. While dimerization of ODC is needed to induce the enzymatic activity, there is no reason to assume that enzymatically inactive AZIN2 would appear as a dimer.

The catalytically active site of ODC is combined of residues from the N terminus of one monomer and the C terminal residues of the other monomer (Tobias KE et al. 1993). The most important residues for ODC activity are K69, C360, and D361 (Tobias KE et al. 1993, Osterman AL et al. 1995, Coleman CS et al. 1993). Variants mutated by these amino acids reveal that each one is required for full catalytic activity. These residues contribute to the binding of the substrate, and K69 is also involved in the binding of the co-factor pyridoxal-5'-phosphate (PLP). C360 appears to mediate the binding of DFMO. The residues associated with the catalytic activity of ODC, K69, E94, K115, K169, H197, D233, E274, D361, and D364 (Osterman AL et al. 1995, Coleman CS et al. 1993, Tsirka S et al. 1992) are conserved in AZIN2, whereas D88 and C360 are not. C360 is conserved in ODC throughout the variety of species, from bacteria to eukaryotes. The ODC mutants C360A and C360S are almost devoid of decarboxylation activity (Tobias KE et al. 1993, Coleman CS et al. 1993). These mutated enzymes, however, catalyze a decarboxylation-dependent transamination reaction to form pyridoxamine-5-phosphate and γ-aminobutyraldehyde, indicating a role for C360 in controlling the specificity of the reaction (Jackson LK et al. 2000). In AZIN2, C360 is replaced by a hydrophobic valine, whereas in AZIN1, C360 is conserved. Biochemical studies have nevertheless shown that both AZINs are devoid of decarboxylating activity on ornithine (Murakami Y et al. 1996a, Kanerva K et al. 2008).

The translocation of ODC to the membrane cytoskeleton is necessary for it to function optimally (Heiskala M et al. 1999). The p47[phox]-like motif, which is responsible for the translocation, is conserved almost entirely in AZIN2. The distribution of AZIN2 has been linked to membrane fractions in mouse brain and to vesicles in mast cells (Lopez-Contreras AJ et al. 2006, Kanerva K et al. 2009). Furthermore, the present results on the localization of AZIN2 in the human brain and testis also suggest that it

is adjacent to membranes. So far, however, there are no data available on the functionality of the p47^{phox}-like motif in AZIN2.

The rapid degradation of ODC in proteasome 26S is induced by binding to AZ (Murakami Y et al. 1992, Murakami Y et al. 1996b). AZ binds to ODC via a specific binding domain (residues 117-140), which is highly homologous in both AZIN2 and AZIN1 (I, Fig. 2). AZINs are capable of displacing ODC from the AZ-ODC complex because of their higher affinity (Snapir Z et al. 2008, Fujita K et al. 1982). AZ binding alone is not sufficient for ODC turnover; two PEST regions in the C-terminus are also needed for the targeting and degradation by proteasome 26S (Li X et al. 1993). The homology between AZINs and ODC decreases towards the C-terminus, and no PEST region is recognized in the C-terminal part of AZINs (http://www.Icnet.uk/LRITu/projects/pest/). The ODC protein is known to be stabilized by truncation of its C-terminus (Ghoda L et al. 1989), suggesting that the C-terminal PEST domains are essential for the turnover of ODC. Furthermore, the electron-dense N- and C-termini of AZIN1 are in closer contact with each other than in ODC, indicating functional differences in these regions (Albeck S et al. 2008).

2.2. Alternative splicing of AZIN2

Alternative splicing is a normal phenomenon in eukaryotes; it increases the diversity of proteins that can be encoded by a limited number of genes. In humans, over 80% of the genes are alternatively spliced enabling encoding of several proteins by a single gene, rather than requiring a separate gene for each polypeptide (Black DL 2003). Alternative splicing has been found to be especially active among genes expressed in human brain (Black DL et al. 2003).

Ten alternatively spliced forms of the coding region of AZIN2 have been detected by sequencing with specific primers to exon I and IX or XI from a QUICKclone cDNA (Clontech) of human brain and testis (II, Table I). The four longest splicing variants (SV) are SV1, 2, 9, and 10, which terminate in exon XI, and are combined of exons as shown in Table 1. A 60-bp extension at the 5´ end of exon VIII and a 84 bp-exclusion

from the 5´ end of exon V distinguish the otherwise identical SV2 and SV9 from SV1, respectively. As compared to SV8, in SV10, exon II includes a 93-bp extension at the 5´end.

Table 1. *The variability of the exon composition of AZIN2. The exons of each splicing variants determined by sequencing the clones are marked (√).*60-bp extension in the 5'-end. **37-bp extension in the 5'-end. † exon length 51-bp (shorter from 5'-end). ‡ 93-bp extension in the 5'-end.*

Splice Variant	Exon I	Exon II	Exon III	Exon IV	Exon V	Exon VI	Exon VII	Exon VIII	Exon IX	Exon X	Exon XI
SV1	√		√	√	√	√	√	√		√	√
SV2	√		√	√	√	√	√	√ *		√	√
SV3	√		√	√	√	√	√	√	√		
SV4	√		√	√	√ **	√	√	√	√		
SV5	√		√		√	√	√	√	√		
SV6	√		√				√	√	√		
SV7	√					√	√	√	√		
SV8	√	√	√	√	√	√	√	√	√		
SV9	√		√	√	√†	√	√	√		√	√
SV10	√	√‡	√	√	√	√	√	√		√	√

Splicing variants 3 through 8 terminate in exon IX: their exon structures are shown in Table 1. Exon IX contains a premature stop codon. This exon is not transcribed in the most abundant RT-PCR product from the brain and testis, and the majority of the ESTs do not include it. Nevertheless, the occasional transcription of exon IX is verified by its presence in rare ESTs from squamous cell lung carcinoma and brain/mixed tissue-derived libraries. By using a 3´ primer designed for exon IX, six different, alternatively spliced variants (SVs 3 through 8) could be amplified from testis tissue.

The sequences of SVs 1–2 and 9–10 were obtained from a pool of human brain mRNA, whereas SVs 1-8 were sequenced from a testis pool. The existence *in vivo* of

the described SVs was not systematically screened in brain and testis, but rather the sequences of different variants emerged during the cloning of AZIN2. Thus, the expression pattern of these variants in different tissues is still to be discovered. Although not specifically tested, the predominance of SV2 and especially of SV1 over the other variants has become apparent through repetitive sequencing of the mRNA pools.

The coding region of AZIN2 SVs 1, 2, 3, 7, 8, and to a lesser extent of SV5, were effectively translated in our system (II, Fig. 1C). Autoradiographic visualization showed approximately the size of 51 kDa for SV2, which agrees with the predicted protein sequences. SDS-PAGE analysis revealed that SVs 1, 3, 7, and 8 showed bands of apparent sizes of 46 kDa, 42 kDa, 40 kDa, and 44 kDa, respectively. As we have not *in vitro* translated the SVs 9 and 10, their translation to proteins *in vivo* remains to be shown. *In silico* translation of the splicing variants implies a premature stop-codon in SV7, whereas the reading-frame of the other variants remains open in spite of alternative splicing.

According to current knowledge, the principal function of AZIN2 is the binding and inactivation of AZ (Kanerva K et al. 2008, Lopez-Contreras AJ et al. 2006, Snapir Z et al. 2008a). The presence of an AZ-binding site is a prerequisite for its function. A comparison of the *AZIN2* sequence to that of the *ODC* gene, in which the AZ-binding site is characterized, indicates the absence of this motif from SVs 5-7 and 9. This implies that these variants of AZIN2 are incapable of binding and inactivating AZs. In addition to the AZ-binding site, the $p47^{PHOX}$ motif was detected from SVs 1–5 and 8–10, although its functional significance in AZIN2 remains to be settled. The splicing variants lacking the AZ-binding site may be futile products of the spliceosome. However, they may also represent non-coding RNAs which are not intended to be translated but rather to regulate the translation process.

The structural homologs and functional counterparts of AZIN2, ODC and AZIN1 are not alternatively spliced to the extent of AZIN2. The long 5'-untranslated region (UTR) of ODC is alternatively spliced to a short form in pancreatic tumor cells enabling an internal ribosomal entry site and fast, cap-independent translation. This

variant of the *ODC* transcript is more sensitive to cell-cycle changes (Pyronnet S et al. 2005).

Two antisera were raised in rabbits to AZIN2 by using the synthetic peptides STRDLLKELTLGASQATTDEVA (antiserum 2) and STRDLLKELTLGASQATT (antiserum 3), corresponding to amino acids 18–39 and 18–35 of AZIN2 sequence (RefSeq Accession NM_052998) as immunogens. The longer peptide spans from exon 1 to exon 3, leaving out exon 2 which is not included in the splicing variants 1–7 and 9. The shorter peptide is encoded only by exon 1, thus being capable of recognizing all splicing variants, including variants 8 and 10 (IV, Fig S1). Another peptide antibody against rodent AZIN2 produced by López-Contreras et al. (2008b) is designed to recognize the boundary between exons 8–10, and therefore leaves out SVs terminating in exon 9.

Two peptide antibodies differed in their ability to detect AZIN2 in different subcellular compartments. In brain, antiserum 3 detected a strong expression of AZIN2 in axons. It also stained pyramidal neurons of the neocortex with a lower intensity. Antiserum 2, in turn, recognized AZIN2 in the soma and apical dendrites of pyramidal neurons but not the axon-localized AZIN2 (IV, Fig 1). Testicular expression of AZIN2 detected by antiserum 3 was concentrated in Leydig cells, and to a lesser extent reacted with spermatogonial cells of seminiferous tubuli (III, Fig 1). Again, the distribution detected by antiserum 2 was more restricted; only spermatogonial cells were stained (unpublished data). It is surmised that this variation in the staining pattern was caused by the selective specificity of the antisera for different splicing variants of AZIN2. López-Contreras et al. (2008b) have reported the expression of AZIN2 in haploid germ cells of mouse testis, but they presented no data on the expression of AZIN2 in mouse Leydig cells. The seemingly discrepant findings on the distribution of AZIN2 in testis obtained with the present antibodies to the N-terminus and the C-terminal antibody of López-Contreras et al. can be explained by their reactivity with different SVs of AZIN2 reflecting biological differences between human and mouse testis.

The presence of several splicing variants of AZIN2 in human brain was demonstrated by RT-PCR with primers to exon I and XI (IV, Fig 2A), and further confirmed by western blotting with antiserum 3 against AZIN2. Two strong bands of AZIN2 were detected from the white matter, while an additional band of apparently larger size dominated in the blottings from the gray matter (IV, Fig 2B).

Taken together, these results indicate that differently spliced variants of AZIN2 occur in the CNS and testis. Furthermore, these data suggest an anatomically divergent distribution of the different splicing variants of the protein. The functional role of these splicing variants of AZIN2 remains to be elucidated, but it is possible that the variants target their expression to specific cellular or sub-cellular distribution.

2.3. Tissue distribution of AZIN2

Whereas ODC is expressed in all types of living cells, with exception for certain archaea, the expression of AZIN2 seems to be restricted to higher eukaryotes (Homo sapiens, Pan troglodytes, Canis lupus familiaris, Bos taurus, Mus musculus, Rattus norvegicus, and Gallus gallus) as evaluated by HomoloGene, NCBI (http://www.ncbi.nlm.nih.gov/homologene/). In Xenopus laevis, the expression of an ODC homolog (XODC2) has been characterized, and that protein is presumably an AZIN2, as judged by its sequence homology (Kidron H et al. 2007). The expression of XODC2 in fetal frog is more restricted than XODC and is located e.g. in the forebrain (Cao Y et al. 2001).

The transcription of *AZIN2* was studied using mRNA Multiple Tissue Expression (MTE) array, RT-PCR, northern blotting, and *in situ* hybridization. With all these techniques, a high expression of AZIN2 was detected in brain and testis. In the MTE array, including samples from altogether 21 specified regions of human central nervous system, expression of AZIN2 was detected widely. Even greater amounts of *AZIN2* transcripts were probed from testis (II, Fig 3). The expression of ODC was detected in all the tissues, although only moderate binding of the probe was seen in the dots from CNS. A similar expression pattern of AZIN2 was described by Lopez-Contreras et al. (2006) in mouse tissues. Using quantitative PCR, they also showed a

predominance of AZIN2 over AZIN1 in mouse brain and testis, suggesting a compensatory regulative role for these homologs (Lopez-Contreras AJ, et al. 2008a). In addition, we have investigated the expression of AZIN2 by northern blotting of total RNA isolated from newborn and adult rat brain and testis. The expression of AZIN2 remained low/moderate in newborns compared to a more abundant expression in adult rat tissues (unpublished data). In mouse testis, the expression of AZIN2 was increasing on postpartum day 22, being nearly absent in newborns and increasing towards adulthood (Lopez-Contreras AJ et al. 2008b). Regunathan et al. (2000) studied the expression of AZIN2 in rat brain, although they falsely called AZIN2 arginine decarboxylase (ADC). They measured the amount of mRNA and protein, and concluded that AZIN2 (ADC in their study) was expressed in all parts of the brain. In accordance with the present results and those of Lopez-Contreras, they showed an increase in the expression of AZIN2 upon neuronal differentiation. The expression data from mRNA samples invariably shows restricted distribution of AZIN2, which differs from the ubiquitous expression of ODC and AZIN1. This further suggests some restriction in the function(s) of AZIN2.

With two validated polyclonal peptide antibodies, the presence of AZIN2 was screened by immunohistochemistry in different parts of brain, testis and some other human tissues. The tissues listed in Table 2 showed positive staining with AZIN2 antibodies.

Table 2. *Human tissues and cell types in which AZIN2 is expressed detected by immunohistochemistry, mRNA dot blot, or in situ hybridization.*

FUNCTIONAL ENTITY	ORGAN
Nervous system	central and peripheral
Reproductive organs	testis, ovary, uterus
Neuroendocrine tissues	adrenal, intestine, neuroendocrine tumors, pancreas, parathyroid, pituitary
Tissues secreting or storaging	breast, brown fat, lung, mast cell, stomach, sweat gland

Immunohistochemical stainings from sections of human testis with the antibodies to AZIN2 revealed robust reactivity with the Leydig cells (III, Fig 1). A subtle granular positive signal was detected in spermatogonial cells. Occasional cells that represented

later stages of spermatogenesis, i.e. spermatocytes, also contained a few positively staining granules. Lopez-Contreras et al. (2008b) found a different distribution of AZIN2 in mouse testis. Their antibody to AZIN2 reacted mainly with haploid spermatids; they did not comment on the expression of AZIN2 in Leydig cells. Since Leydig cells were not visible in their micrographs, the estimate of the species-dependent differences in the expression of AZIN2 is difficult to interpret. Furthermore, Lopez-Contreras et al. did not show any validation of their peptide antibody to mouse AZIN2. Interestingly, the interstitial Leydig cells in mouse testis have been found to express considerably higher ODC activity than the proliferating spermatogenic cells (Qian ZU et al. 1985) suggesting that it acts as an ODC inductor in these cells.

Ovarian luteal cells produce steroid hormone similarly to the Leydig cells in testis. Immunohistochemical staining of sections from the ovaries of fertile women showed a strong expression of AZIN2 in luteal theca cells lining corpus luteum cysts (III, Fig 2). No positive staining was seen in corpora albicantia or granulose cells of corpus luteum. Stainings of sections from postmenopausal ovaries revealed a strong positivity in the ovarian hilus cells which are responsible for progesterone synthesis.

Intact ODC activity is needed for normal folliculogenesis and luteinization in mouse ovary (Bastida CM et al. 2005). Moreover, blocking of the preovulatory rise of ODC activity by *in vivo* treatment with DFMO abolished the secretion of progesterone by the corpus luteum (Bastida CM et al. 2002). Testosterone-producing Leydig cells of mature murine testis maintain high ODC activity (Qian ZU et al. 1985), whereas the ODC activity is lower in Sertoli and germ cells of adult rodents (Shubhada S et al. 1989). Treatment with gonadotropins induces rapidly upregulated ODC activity in rat ovaries and testis (Osterman J et al. 1983, Maudsley DV et al. 1974). The gonodatropin-induced activation of ODC in gonads suggests that ODC has a role in the regulation of steroid hormone secretion or synthesis. ODC has a very short half-life, and its *de novo* synthesis and release from an inactive complex with AZ is considered to occur in response to a stimulus, thus requiring the activity of AZIN. According to the results, AZIN2 could function as a part of a regulative machinery of ODC activity.

In human and murine testis, a testis-specific AZ, AZ3, is expressed, and the preliminary hypothesis was that ODC, AZ3, and AZIN2 form a regulatory triangle in polyamine synthesis in testis. The *in situ* hybridization studies of sections of normal human testicular tissue with an AZ3 probe nevertheless revealed a positive signal only in spermatids of the seminiferous tubules (III, Fig 3a) as previously shown in murine testis (Lopez-Contreras AJ et al. 2008b). Thus, no expression of AZ3 was detected in Leydig cells, showing that AZIN2 and AZ3 are mainly expressed in different cells of the human testis. Instead, expression of AZ1 has previously been reported to occur in both Leydig cells and Sertoli cells (Tosaka Y et al. 2000). López-Contreras et al. (2008b) have reported co-expression of AZIN2 and AZ3 in haploid germ cells of mouse testis. As mentioned above, it remains to be clarified whether the seemingly discrepant findings reflect biological differences between human and mouse testis, or differences of antibodies to detect alternatively spliced forms of AZIN2. In conclusion, the interaction between AZ3 and AZIN2 in haploid germ cells still needs to be studied further. Since no expression of AZ3 in Leydig cells or alternative splicing has been detected, it is likely that AZ1, rather than AZ3, is a regulatory counterpart of AZIN2 in Leydig cells.

Despite its high expression in Leydig cells and ovarian luteal cells, Sertoli cells in the normal seminiferous tubules remained unstained with antibodies to AZIN2. This applied also to hyperplasic Sertoli cells in testis biopsies from patients with germ cell aplasia or Sertoli cell-only syndrome. These results indicate that AZIN2 is not a general marker for endocrine cells in the gonads. The expression of AZIN2 was also investigated in testicular neoplasms derived from either Leydig cells or germinal epithelium, and in ovarian thecomas. All of the Leydig cell tumors studied (n=4) stained positively for AZIN2 (III, Fig 1g, h). In addition, a scattered granular staining of AZIN2 was seen in ovarian thecomas (III, Fig 2d). Instead, no staining for AZIN2 was seen in neoplasms derived from the germinal epithelium (seminomas (n=5), yolk-sac tumors (n=2), or embryonal carcinomas (n=3), data not shown). However, as far as can be quantified from immunohistochemistry, the expression level of AZIN2 was lower in the neoplastic than in normal Leydig cells. The expression of AZIN2 seems to be restricted to more mature and terminally differentiated cells. The same observation was made when comparing the levels of AZIN2 mRNA in the brain and testis of newborn versus adult mice.

Different parts of the human brain were studied immunohistochemically for the expression of AZIN2 with two peptide antibodies (IV, Table 1). The neuronal tracts were strongly visualized in all studied areas. Axon bundles emerging from lamina V–VI were strongly positive in the neocortex and white matter (IV, Fig 1). Also dendrites in the subpial plexus were visualized. In the hippocampus, AZIN2 was detected in axons of the dentate gyrus, of the perforant pathway, alveus, and entorhinal cortex. In the cerebellum, the axons of basket cells and Purkinje cells expressed AZIN2. Also the tracts of the thalamus and medulla oblongata showed abundant reactivity. Staining of the temporal and frontal lobe visualized AZIN2 also in the soma of medium and large pyramidal neurons in lamina III–V. However, the expression of AZIN2 in the somas of pyramidal neurons differed between adjacent areas in the neocortex, giving an impression of spatial and/or temporal regulation of AZIN2 expression. This might implicate a selective expression of AZIN2 in cells with certain neurotransmitters, or as a response to neuronal activation. *In situ* hybridization further demonstrated the regional distribution of cells expressing AZIN2 mRNA. In addition to the neocortex, somas of some neurons in the medulla oblongata and Purkinje and basket cells in the cerebellum also expressed AZIN2. Neurons of the hippocampus, basal ganglia, and thalamus expressed only minor reactivity with antiserum 2. In the sections from the temporal lobe, AZIN2 was occasionally detected in the cytoplasm of oligodendrocytes. Kindling and other strong electrical stimuli evokes also expression of ODC in glial cells {{3 Bernstein,H.G. 1999; }}. In accordance with the expression pattern and cell type specificity of AZIN2 determined from testicular tissues, this protein functions only in terminally differentiated cells. Considering the variety of cellular functions in which ODC and polyamines participate, it seems likely that their inductors AZIN1 and AZIN2 not only are differently distributed but also replace each other in the regulation of different cellular phenomena. It is noteworthy that AZIN1 and AZIN2 are also expressed simultaneously in certain cell types, including neurons and Leydig cells. This fact further emphasizes the selectivity in their regulatory functions. The distribution in different subcellular compartments could provide a mechanistic way to elucidate the relative roles of AZIN1 and AZIN2 in the regulation of ODC.

2.4. Subcellular distribution of AZIN2

In order to understand the biological role of AZIN2, its intracellular localization is of fundamental importance. The current knowledge of the distribution of AZIN2 in subcellular compartments is derived from immunohistochemical stainings and cell fractionation studies.
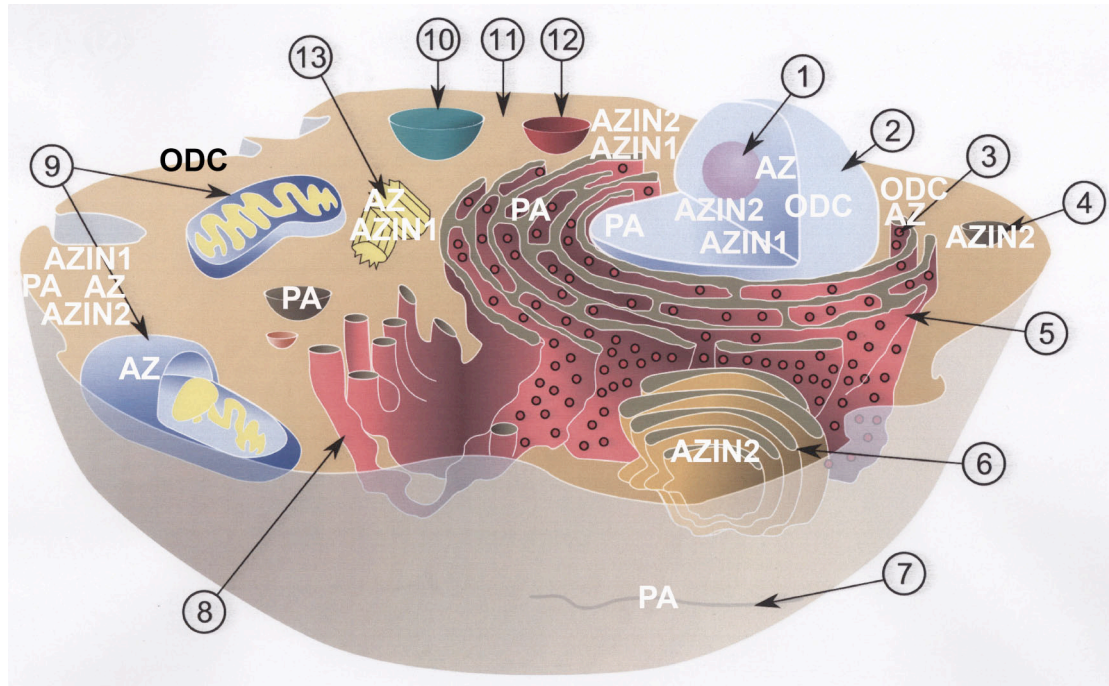

Figure 4. *Antizyme (AZ), antizyme inhibitor 1 and 2 (AZIN1-2), ornithine decarboxylase (ODC), and polyamines (PA) are localized in various intracellular organels. 1. nucleolus, 2. nucleus, 3. ribosome, 4. vesicle, 5. rough endoplasmic reticulum, 6. Golgi apparatus, 7. cytoskeleton, 8. smooth endoplasmic reticulum, 9. mitochondrion, 10. vacuole, 11. cytosol, 12. lysosome, and 13. centriole. The original figure of the morphological cell was produced by Messer and Szczepan (Wikipedia, cc-by-sa).*

Immunohistochemical stainings invariably show a vesicular or granular distribution of AZIN2 in human cells. In the somas of neurons, these vesicle-like structures which contain AZIN2 were visualized in the soma close to the plasma membrane (IV, Fig 3A). In axons, AZIN2 was also observed in granular or vesicle-like structures along the processes (IV, Fig 3B). As in the somal area, AZIN2 was localized close to the inside of the plasma membrane following the outer boundaries of the neurite. The vivid traffic of neurosecretory granules along the axonal cytoskeleton depends greatly

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on the activation of small G proteins (for a review, see Ng EL et al. 2008). Given the present results on the regulation of cytoskeleton dynamics via the polyamination of a G protein RhoA (I), it is tempting to speculate that AZIN2 might regulate ODC-dependent local polyamination of also other G proteins involved in the traffic of neurosecretory vesicles.

A distinct granular distribution of AZIN2 was found in the lateral neurons of the medulla oblongata (area of spinal trigeminal and ambiguous nuclei) and the Purkinje cells of cerebellum. This staining was not restricted to the vicinity of the plasma membrane, but was rather dispersed throughout the cytoplasm and along the apical dendrite. A similar vesicular distribution of AZIN2 was detected in several types of endocrine and secreting cells (Fig 5), e.g. in the Leydig cells and spermatogonia of testis (III, Fig 1). The vesicular distribution of AZIN2 has also been clearly demonstrated in mast cells. The vesicle traffic of serotonin granules was linked to the distribution and expression of AZIN2 as well as to the catalytic activity of ODC (Kanerva K et al. 2009).

Figure 5. *The expression of antizyme inhibitor 2 in human sweat gland detected by immunohistochemistry. The glandular parts show high vesicle-like expression whereas in ductal parts (indicated by arrowheads) the expression is low or absent.*
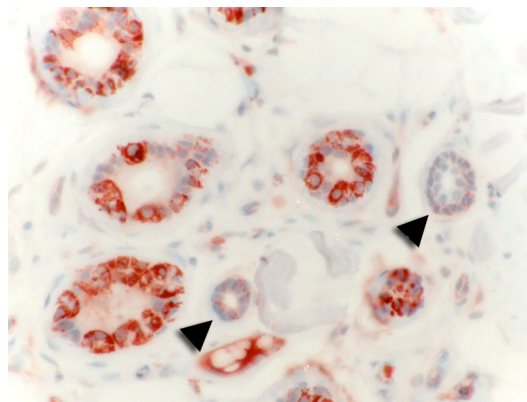
The association of AZIN2 with membranes was demonstrated from membrane fractions of cultured cells transfected with flag-tagged AZIN2 (Lopez-Contreras AJ, et al. 2006). No mitochondrial localization of AZIN2 could be detected by our doublefluorescent stainings with antibodies to AZIN2 and MitoTracker (Molecular Probes) (Kanerva K et al. 2008) even though this has been suggested earlier (Lopez-Contreras AJ et al. 2006). When epitope-tagged AZIN2 localization was studied in more detail, AZIN2 localized to the ER-Golgi intermediate compartment and the *cis*-Golgi network (Lopez-Contreras AJ et al. 2009). This further emphasizes that AZIN2

is likely associated with vesicle trafficking. However, the subcellular localization of AZIN2 from transfected cells has to be interpreted with caution since overexpression due to efficient transfection might saturate the cellular compartments and contort the intracellular distribution. An additional epitope linked to a protein may also influence the subcellular distribution.

There are only a few studies on the intracellular distribution of AZIN1. It is considered, however, to be diffusely distributed over the cytoplasm and is also found in the nucleus (Lopez-Contreras AJ et al. 2009). The nuclear localization fits with its function in the regulation of cell proliferation. Interestingly, we have occasionally observed nuclear staining of also AZIN2 in tissue specimens detected by immunofluorescence and immunohistochemistry and also more frequently in cultured cells transfected with AZIN2 (IV, Kanerva K et al. 2009, and our unpublished data).

2.5. Functional implications of AZIN2

AZIN2 has no known catalytic activity, and functions inside a cell in regulating the concentration of polyamines. Although less efficiently than AZIN1, AZIN2 binds the ODC inhibitor AZ and liberates ODC monomers, enabling the formation of catalytically active homodimers of ODC (Kanerva K et al. 2008, Snapir Z et al. 2008a). In addition, AZIN2 increases the uptake of polyamines to the cell (Lopez-Contreras AJ et al. 2008a). The amount of AZ is controlled independently of AZIN: the transcription is upregulated by high a polyamine concentration, and AZ is ubiquitinated to signal degradation (Gandre S et al. 2002). Nevertheless, AZIN1 and 2 may function as local inductors of polyamine synthesis in selected subcellular compartments.

AZIN1 is distributed ubiquitously, and its expression is stimulated by mitotic signaling (Nilsson J et al. 2000). It is also highly expressed in certain cancers, indicating a role in cell transformation and malignant growth (Jung MH et al. 2000, Schaner ME et al. 2005, Keren-Paz A et al. 2006). Instead, AZIN2 expression was low or absent in cancer cell lines (II, Fig 3) and it is less potent in stimulating growth (Snapir Z et al. 2008a). Our results show high expression of AZIN2 in terminally differentiated cells, including neurons, Leydig cells, luteal ovarian cells and mast

cells, implying that it is evidently not connected with cell proliferation. It thus appears that AZIN1 and AZIN2 regulate ODC for different purposes (III, IV, Kanerva K et al. 2009).

In the steroid-hormone-producing cells of testis and ovary, the activity of ODC is stimulated by luteinizing hormone (LH) (Osterman J et al. 1983, Maudsley DV et al. 1974). Analogously, in the adrenal, where we have detected AZIN2 expression, adrenocorticotropin stimulates the activity of ODC (Levine JH et al. 1973). Furthermore, DFMO-mediated inhibition of LH-induced stimulation of ODC activity dramatically decreased the production of progesterone in mouse corpus luteum (Bastida CM et al. 2002). These observations indicate that polyamines and ODC have an important role in the regulation of steroid hormone synthesis and/or release. Evidence for a connection between the AZIN2-polyamine system and cellular secretion was also obtained from mast cells. These cells contain large granules which are released upon stimulation. The intracellular localization of AZIN2 to vesicle-like structures proceeded along with granule movement, and its expression was elevated also in degranulating mast cells (Kanerva K et al. 2009). Inhibition of ODC by DFMO prevented the release of serotonin granules. These findings suggest that AZIN2 has a functional role in mast cells in vesicle movement as a regulator of polyamines.

Polyamines and ODC are related to the functional regulation of ion channels in the CNS. The inward rectifier K^+ channels that regulate membrane potential and cell excitability are blocked by polyamines (Fakler B et al. 1994, Ficker E et al. 1994, Lopatin AN et al. 1994). Polyamines also inhibit the AMPA- and kainate-dependent fast depolarization of glutaminergic synapses, and thereby modulate neurotransmitter signals (Kamboj SK et al. 1995, Bowie D et al. 1995). The N-methyl D-aspartate type excitatory glutamate receptors (NMDAR) form glutamate-gated ion channels in the brain and mediate synaptic plasticity which is pivotal for cognitive functions, such as memory and learning (Bliss TV et al. 1993). NMDARs are regulated by polyamines in multiple ways: the binding of polyamines from the extra- or intracellular side to NMDAR can either enhance or inhibit the influx of Ca^{2+} (Araneda RC, et al. 1999, Benveniste M et al. 1993, Durand GM et al. 1993). Pretreatment of cultured mouse cortical neurons with DFMO abolished NMDA-induced neurotoxicity (Markwell MA

et al. 1990). This indicates that intracellular levels of polyamines are involved in the functioning of NMDAR. Since AZIN2 regulates polyamines, double immunofluorescence was used to investigate the interrelationship between AZIN2 and NMDAR. All the cells expressing AZIN2 were also positive for NMDAR1, even though NMDAR1 was more widely distributed, and AZIN2 showed a more restricted pattern of expression. This demonstrates overlapping localization of AZIN2 and NMDAR1 in the cytoplasm of large pyramidal cells in the neocortex of normal brain (IV, Fig 4). Although the molecular details are largely unknown, the local regulation of the ODC activity and the concentration of polyamines are obviously important for the functioning of the channels and for neurotransmitter signalling. In biochemical assays, AZIN2 not only stimulates the intracellular polyamine synthesis, but also regulates the local extracellular concentration by enhancing the uptake of polyamines (Kanerva K et al. 2008, Lopez-Contreras AJ et al. 2008a). NMDARs circulate constantly between the synaptic plasma membrane and cytoplasmic vesicles upon neuronal activation and sensory experience, thereby contributing to long-term potentiation (Grosshans DR et al. 2002, Quinlan EM et al. 1999). Vesicles containing NMDAR are transported along microtubules to the postsynaptic density and extrasynaptic areas (Washbourne P et al. 2002, Tovar KR et al. 1999). Given that polyamines regulate NMDAR, AZIN2 might influence glutamate-mediated signalling by controlling the local synthesis of polyamines.

Most of the ODC in the brain is bound to antizyme which blocks its enzymatic activity (Laitinen PH et al. 1986, Laitinen PH 1985, Laitinen PH et al. 1985). However, various stimuli, including electrical and chemical stimulation, and traumatic injuries, activate ODC (Pajunen AE et al. 1978, Bondy SC et al. 1987, Martinez E et al. 1991, Baskaya MK et al. 1996, Dienel GA et al. 1984). Elevated ODC activity and concentration of putrescine have been detected in the brain in pathological conditions such as ischemia and Alzheimer's disease (AD) (Lukkarinen JA et al. 1999, Keinänen R et al. 1997, Bernstein HG et al. 1995, Morrison LD et al. 1995, Morrison LD et al. 1998, Paschen W et al. 1991). In AD, the pathological accumulation of β-amyloid induces activation of ODC, in addition to its ability to provoke the generation of neurotoxic reactive oxygen species (Nilsson T et al. 2006b, Behl C et al. 1994, Hensley K et al. 1994). Putrescine functions as a scavenger of free

radicals and thereby buffers the cytotoxic effects of ROS (Yatin SM et al. 2001). Yatin et al. have further shown that β-amyloid, in addition to inducing ODC activity, also stimulates polyamine uptake (Yatin SM et al. 1999). The cellular uptake of polyamines and the induction of ODC activity are regulated by AZINs. The expression of AZIN2 in 15 human brains affected by AD was studied in sections from the frontal lobe and the hippocampus (IV, Fig 5). The regional variation of AZIN2 expression seen in normal brain was abolished and, instead, virtually all the pyramidal cells in the frontal cortex stained positively for AZIN2. The individual cells of the AD brains also appeared to contain larger amounts of AZIN2. In the pyramidal cells, AZIN2 was seen in large aggregates or in vacuoles. The hippocampal regions CA1-3 of AD brains displayed a robust expression of AZIN2 that was not found in samples of normal brain. In the axons, which appeared swollen, AZIN2 was accumulated in vacuole-like aggregates that were larger than those seen in the axons of normal brain tissue. The subcellular localization of ODC has also been reported to be influenced by AD. Nilsson et al. found evidence for translocation of ODC from the nucleus to the cytoplasm of neocortical pyramidal cells in the early phase of AD. In addition, elevated expression of ODC was found in Purkinje cells of the cerebellum and in the hippocampus of AD brains (Nilsson T et al. 2006a). Elevated expression of AZIN2 was also detected in Purkinje cells and the hippocampus in AD. However, earlier reports on the immunohistochemical location of ODC have to be considered with caution since the antibodies used were raised before the identification of AZIN2. They may therefore have caused substantial cross-reactivity due to the high degree of structural homology between ODC and AZIN2. The accumulation of AZIN2 in AD may nevertheless provide neuroprotection by enhancing the production and uptake of putrescine stimulated by β-amyloid.

The expression of AZIN2 in the brain was not increased in the other neurodegenerative diseases studied. In the samples from patients with CADASIL dementia and from patients with dementia with Lewy bodies (DLB), the expression of AZIN2 was similar to that seen in samples from the corresponding areas of normal brain. The neuronal accumulation of AZIN2 is therefore merely a feature of AD. Polyamines, and in particular spermine, have been associated with the pathogenesis of Lewy body dementia and Parkinson's disease. The binding of spermine to α-

synuclein provokes its folding and leads to the formation of aggregates (Grabenauer M et al. 2008). The aggregation of α-synuclein is considered to be a major pathological event in these neurodegenerative diseases. It would be of interest to study whether yet uncharacterized splicing variants of AZIN2 are related to Parkinson's disease, or whether there is an accumulation of AZIN1 in these pathological states.

CONCLUSIONS AND FUTURE PERSPECTIVES

A novel molecular mechanism was introduced by which ODC and polyamines regulate the reorganization of actin cytoskeleton. The small G protein RhoA is polyaminated by transglutaminase, leading to its activation. Activated RhoA induces changes in cytoskeletal dynamics. Polyamination of RhoA occurs physiologically during mitosis, and explains why polyamines are needed for the cell cycle to the progress from mitosis to G1. During cytokinesis, active RhoA is known to accumulate in cleavage furrow and to regulate the contractile actin-myosin fibers in the mitotic spindle.

Polyamine synthesis is not the only way by which ODC controls the functioning of RhoA. The membrane translocation of ODC, which is crucial for its transforming capacity, either directs RhoA to the vicinity of the plasma membrane, or is needed for the local maintenance of active RhoA. It is of importance since RhoA is active in the membrane and inactive in the cytoplasm.

Constitutive overexpression of ODC leads to the transformation of NIH 3T3 cells, and high activity of ODC has been observed in many cancers. Cytoskeletal rearrangements are a major event in proliferation, attachment, and migration of cells. Thus, they play a crucial role in transformation and the behaviour of malignant cells. The results provide a mechanistic link between polyamines and the actin cytoskeleton, and help in understanding how polyamines influence cell transformation. In the future, it would be interesting to evaluate the polyamination status of RhoA in different types of malignant cells and to test the tumor promotion and metastasizing capacity of cells in which the polyamination of RhoA is manipulated.

A new member has been characterized in the polyamine-regulative family in addition to ODC and AZIN1. This novel protein was devoid of ODC activity and was termed as AZIN2. The expression of AZIN2 was mainly found in terminally differentiated cells including neurons and Leydig cells. This suggests that the role of AZIN2 differs

from that of AZIN1, which is implicated in the regulation of growth and proliferation. Another distinction from AZIN1 is the extensive alternative splicing of AZIN2 transcripts. It appears evident that there is selective expression of variants between cell types and even subcellular compartments. However, the ultimate biological significance of these remains to be determined.

Immunohistochemical stainings of sections from different human tissues appoint to their correlation with vesicle function. The high expression of AZIN2 in testosterone-producing Leydig cells and progesterone-secreting luteal cells of male and female gonads, respectively, suggest an association with secretion and vesicle function. Future aims will be focussed more closely on the vesicles to which AZIN2 localizes, and the role of AZIN2 in vesicle function will be investigated.

In brain, polyamines regulate NMDA-type glutamate receptors responsible for excitatory signalling, as well as Kir channels which monitor the threshold for action potentials. As AZIN2 colocalizes with NMDAR1 in human brain, it would be of interest to study whether AZIN2 serves as a local inductor of polyamine synthesis in the vicinity of glutamate receptors and in this way regulates their function. An accumulation of AZIN2 in neurons was detected in AD. Whether it is a consequence of pathological changes in neurons or a part of pathogenesis itself remains to be clarified. However, this result emphasizes the importance of studying further the role of polyamines and ODC in AD. However, the accumulation of AZIN2 is not a general response to neurodegeneration, since no accumulation was detected in neurons from patients with CADASIL or dementia with Lewy bodies.

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