Molecular mechanisms underlying morphological effects of protein kinase C under normal conditions and cellular stress.

Sunesson, Lovisa

2008

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Sunesson, L. (2008). Molecular mechanisms underlying morphological effects of protein kinase C under normal conditions and cellular stress. Lund University, Faculty of Medicine
Abstract
The protein kinase C (PKC) family of serine/threonine kinases consists of 13-15 members. The PKC isoforms have central roles in many essential cellular processes, including proliferation, apoptosis, differentiation, cytoskeletal changes, and migration. The PKC family can be subgrouped into classical, novel and atypical isoforms depending on structure and sensitivity to the activators diacylglycerol, phorbol esters and Ca²⁺.

We have previously reported of a neurite-inducing effect specific for PKCa, a novel PKC isoform. The effect has been shown to be mediated through the regulatory domain and independently of the catalytic activity of PKCα. Here we identify residues located in the base of the C1b domain important for the neurite-inducing effect of PKCα. We have in an approach to elucidate the mechanisms that mediate PKCα-induced neurite outgrowth performed a screening for proteins that interact with the most potent neurite-inducing PKCα construct. We have identified several novel PKCα-interacting proteins, including the intermediate filament peripherin and several mRNA-binding proteins. Peripherin and three RNA-binding proteins G3BP2, PABPC1, and IGF2BP3 were further investigated regarding their putative function in PKCα-mediated neurite outgrowth. However, no involvement could be detected for any of the investigated interacting proteins. Interactions between peripherin, G3BP2, PABPC1, or IGF2BP3 and PKCα have been confirmed for endogenous proteins. The peripherin-PKCα interaction is mediated by the C1b domain. We have found that PKCα induces peripherin aggregation when the expression levels of peripherin were elevated and that activation of PKCα induces apoptosis of cells overexpressing peripherin. These novel findings can be of importance in understanding the mechanism behind the neurodegenerative disease amyotrophic lateral sclerosis (ALS), where aggregates containing peripherin are essentially always seen in afflicted tissues. The RNA-binding proteins were found to localize to specific RNA-granules, formed when cells are exposed to stress. PKCα, rather than PKCα, was found to be the main PKC isoform found in these stress granules. We have furthermore discovered a role for PKCα in regulating stress granule formation.

Key words: Protein Kinase C, neurite outgrowth, protein-protein-interactions, peripherin, amyotrophic lateral sclerosis, RNA-binding proteins, G3BP2, PABPC1, IGF2BP3, neurobiotaxis

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This thesis is based on the following papers, which are referred as Papers I-III:

I

II

III
Lovisa Sunesson*, Tamae Kobayashi*, Ulf Hellman, and Christer Larsson. PKCα associates with the RNA-binding proteins PABPC1, IGF2BP3, and G3BP2 and regulates stress granule formation following cellular stress. Manuscript.
* These authors contributed equally to this work

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Introduction

I was first introduced to protein kinase C (PKC) in January of 2003. Of course, I had probably read about the kinase during my previous studies, but never really thought anything special about it. In the beginning of 2003 I had reached the end point of my master’s degree and it was time for doing my master’s thesis. I began working with PKC, more exactly with PKCepsilon (ε), and initiated what would become an interesting journey in the world of PKC. I began investigating the mechanisms behind PKCε-mediated neurite outgrowth but somewhere on the way my results led me in other directions.

The protein kinase C family of proteins was discovered in the late seventies (1) and named "C" after calcium. The family consists of serine/threonine protein kinases that phosphorylate or catalyze the transfer of phosphate from ATP to serine or threonine residues on their substrates. The family is divided into three groups, depending on their sensitivity to different co-activators such as the lipid second messenger diacylglycerol (DAG), phorbol esters (functional analogs of DAG) and Ca$^{2+}$, called classical, novel, and atypical PKCs. The sensitivity for Ca$^{2+}$, phospholipids and DAG was discovered by the same group that discovered PKC (2,3).
The Protein Kinase C

The PKC molecule consists of one amino(N)-terminal regulatory half and one carboxy(C)-terminal catalytic or kinase half separated by a proteolytically labile hinge region (V3) (Figure 1). The catalytic domain is very similar to that of protein kinase A and protein kinase B (also known as Akt) (4-6). The PKC polypeptide consists of four conserved domains called C1 to C4, where each domain confers a special function to the protein, and five variable (V1-V5) domains positioned between the conserved domains (7-10). 12 PKC isoforms have so far been identified and several isoforms have so far been identified and several PKCs (17,18). The classical PKCs (15), the conventional PKC isoforms, first identified (10,13), include α, βI and βII (products of alternative splicing) and five variable (V1-V5) domains positioned in-between the conserved domains (7-10). PKCs (17,18).

The PKC Structure

Elucidating the structure of the intact full-length PKC molecule has been difficult, whereas the structure of individual domains, such as the two membrane-binding domains C1 and C2, have been easier to determine (19-27). Recently the relationships between C1a, C1b, and C2 domains and the membrane or between individual domains and the catalytic domain were studied in an intact PKCδ crystal (28).

The Pseudosubstrate

The pseudosubstrate is identical in all classical PKC isoforms and quite conserved among other isoforms and functions as an autoinhibitory domain by interacting with the C4 domain and maintaining PKC in an inactive state (7-9, 30). The pseudosubstrate was the first sequence shown to be involved in an intramolecular inhibitory interaction in PKC (29). The pseudosubstrate domain insensitive to DAG/phorbol esters and no C2 domain (6,7). Additional kinases have been proposed to belong to the PKC family. The protein kinase C-related kinase (PRK) family of at least 3 proteins (PRK1-3) is one such example (12,16). Another example is the phosphatidyl-dependent kinase called PKD/PKCµ, which shows close homology to the regulatory domain of the novel PKCs (17,18).

The Protein Kinase C

The PKC molecule consists of one amino(N)-terminal regulatory half and one carboxy(C)-terminal catalytic or kinase half separated by a proteolytically labile hinge region (V3) (Figure 1). The catalytic domain is very similar to that of protein kinase A and protein kinase B (also known as Akt) (4-6). The PKC polypeptide consists of four conserved domains called C1 to C4, where each domain confers a special function to the protein, and five variable (V1-V5) domains positioned in-between the conserved domains (7-10). 12 PKC isoforms have so far been identified and several isoforms have so far been identified and several PKCs (17,18). The classical PKCs (15), the conventional PKC isoforms, first identified (10,13), include α, βI and βII (products of alternative splicing) and five variable (V1-V5) domains positioned in-between the conserved domains (7-10). PKCs (17,18).

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with the negatively charged phospholipids in the membrane creating a contiguous hydrophobic surface without any conformational change (7,9,27,35-37). The penetration into the membrane and the following formation of a hydrophobic surface stabilizes the C1-membrane complex (38). The middle region of the C1 domain mainly contains positively charged residues that interact with the negatively charged phospholipids in the plasma membrane (32).

The C1a and C1b domains have long been under investigation and it has been found that usually only one of the repeats bind DAG/phorbol esters and that the binding affinity for the C1 ligands differs between individual C1 domains and between C1 domains of different PKC isozymes (39-42). The atypical C1 domain differs in the structure compared to the typical C1 domains in that they lack the hydrophobic residues at the top of the C1 domain. In addition, the structure of the atypical C1 domain is not as flexible as the typical C1 domains making them unable to form a binding pocket (43,44). They are also more positively charged due to four extra arginine residues at the top of the C1 domain (45). PKCs are not the only proteins having C1 domains. Other proteins have been identified containing a C1 domain and the first one to be identified was n-chimera later renamed n1-chimera (46). Additional examples of C1-containing proteins binding DAG, are members of the PKD family and the DAG kinase (DGK) family, and the Munc13 family of scaffolding proteins (47,48). Many, however, of these proteins lack a kinase domain and are therefore called “non-kinase” phorbol ester receptors (49).

The C2 Domain

The C2 domain is about 130 amino acids long and consists of eight anti-parallel β strands. It contains a recognition site for acidic phospholipids, such as phosphatidylserine, and, in classical PKC isoforms, a Ca²⁺-binding aspartate-lined pseudosubstrate consensus sequence except that it has an alanine instead of a phosphorylatable serine/threonine residue in its sequence. The pseudosubstrate consequently binds to the substrate binding site in the C4 domain without triggering the PKC molecule to the membrane, in all but the atypical PKCs (31) and depending on their ability to bind DAG/phorbol esters they are divided into either typical or atypical C1 domains. The C1 domains can also be divided into exposed and non-exposed C1 domains referring to the location of the C1 domain in the native full-length protein (32,33).

The C1 Domain

The C1 domain consists of roughly 50 amino acids. It is often duplicated and the individual C1 domains have been termed C1a and C1b. The C1 domains bind diacylglycerol and phorbol esters, targeting the PKC molecule to the membrane, and the following formation of a hydrophobic groove that forms a ligand-binding hydrophilic groove (38). The middle region of the C1 domain mainly consists of eight anti-parallel β strands. It contains a recognition site for acidic phospholipids, such as phosphatidylserine, and, in classical PKC isoforms, a Ca²⁺-binding aspartate-lined pseudosubstrate consensus sequence except that it has an alanine instead of a phosphorylatable serine/threonine residue in its sequence. The pseudosubstrate consequently binds to the substrate binding site in the C4 domain without serving as a substrate (30). Upon binding of DAG or phorbol esters to the C1 domain, a conformational change of the PKC molecule leads to a relief of autoinhibition.

FIGURE 2. 3-dimensional structure of the C1b domain, C2 domain (with bound Ca²⁺), and kinase domain. Adapted from (6).
cleft (Figure 2) (9,35,37). These aspartates are not present in C2 domains that do not bind Ca2+—explaining why novel PKCs are calcium-insensitive and have low sequence homology to the C2 domains of classical PKC isoforms (51). Ca2+ functions as a link between the C2 domain and anionic phospholipids by inducing a conformational change of the C2 domain that enables the C2 domain to bind phospholipids and by increasing the affinity for negatively charged lipids (35,52). The classical PKC isoforms can be differentially regulated by changing the amount of calcium released upon receptor stimulation (53).

Many other proteins also contain C2 domains and when comparing C1 and C2 domains the C2 domains show much more functional diversities (12,35). The C2 domains are also involved in both intra- and intermolecular interactions important for PKC activation, translocation, substrate-binding and anchoring (54). Interactions between C1 and C2 domains of different PKC isoforms have been shown to stabilize and activate the PKC isoforms involved in the interaction (55).

The C3 and C4 Domains

The C3 and C4 domain together constitute the catalytic domain and as much as 60% of this domain is shared between the different PKC isoforms (Figure 2) (56). The C3 domain contains the ATP-binding site and this site is the target for most PKC inhibitors (7,8). Replacing a conserved lysine residue in the ATP-binding site leads to a catalytically inactive kinase (57). The C4 domain contains the active site or the substrate-binding site and one of three PKC-regulatory phosphorylation sites, the autophosphorylation site (7,8).

The V5 Region

The V5 region is very important in regulating the kinase activity of PKC, since it is here the two remaining phosphorylation sites regulating PKC activity are located (12). The V5 region contains a nuclear localization sequence (NLS) and is, like so many other PKC domains, also involved in intramolecular inhibitory interactions (58,59).

The PKC Regulation

Binding of DAG/Phorbol Esters and Ca2+

Ligand-binding to G protein coupled receptors such as growth factor, neurotransmitter or hormone receptors, tyrosine kinase receptors, and nonreceptor tyrosine kinases leads to PKC activation via two mechanisms involving either phospholipase C (PLC) or phospholipase D (PLD) (Figure 3). PLC mediates hydrolysis of inositol phospholipids such as phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) yielding DAG and inositol 1,4,5-trisphosphate (IP3), which causes release of intracellular Ca2+. PLD mediates hydrolysis of phosphatidylinoholcholine, the principal phospholipid in the plasma membrane, yielding choline and phosphatic acid, which in turn is converted into DAG by phosphomonoesterase (9,11). The DAG metabolism differs between the two different DAG products. DAG produced by PI(4,5)P2 hydrolysis is rapidly converted into phosphatic acid by DGK, whereas DAG produced from phosphatidylinoholcholine is slowly degraded by a specific lipase (60).

Upon activation, classical and novel PKCs translocate from the cytosol to the membranes by binding to DAG/phorbol esters. DAG/phorbol esters serve as hydrophobic anchors to recruit PKC to the membranes as well as enhancing the affinity of PKC for phosphatidylinosine by decreasing the Ca2+ concentration needed for phosphatidylinosine-binding (31,61-63). For PKC to become fully activated the pseudosubstrate cleft (Figure 2) (9,35,37). These aspartates are not present in C2 domains that do not bind Ca2+—explaining why novel PKCs are calcium-insensitive and have low sequence homology to the C2 domains of classical PKC isoforms (51). Ca2+ functions as a link between the C2 domain and anionic phospholipids by inducing a conformational change of the C2 domain that enables the C2 domain to bind phospholipids and by increasing the affinity for negatively charged lipids (35,52). The classical PKC isoforms can be differentially regulated by changing the amount of calcium released upon receptor stimulation (53).

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PKC Phosphorylation
PKC is, in addition to ligand interactions, also regulated by phosphorylation (67). PKC has been shown to be autophosphorylated in vitro (68,69) and phosphorylated on at least three sites in vivo (Figure 2) (70,71). The first step includes transphosphorylation of a residue located in the kinase activation loop critical for enzyme activation (70-73). This phosphorylation site is thought to be hidden when the pseudosubstrate is bound to the active site and needs to be exposed to become phosphorylated (70). The second and third steps involve autophosphorylation of two C-terminal residues termed the turn motif and hydrophobic site. The hydrophobic site is present in many other kinases (70,71). However, phosphorylation of PKC only primes PKC during protein maturation and PKC still needs to interact with second messengers to become fully activated. Most PKC isoforms in resting cells are phosphorylated on all three phosphorylation sites, speaking for the phosphorylation events only being part of the PKC maturation and not in PKC regulation (9).

PKC Activators
Phorbol esters were shown in the early eighties to activate and translocate PKC to different cellular compartments (80) and have since been extensively used as PKC activators (7). 12-O-tetradecanoylphorbol-13-acetate (TPA) or phorbol 12-myristate 13-acetate (PMA) is the most frequently used phorbol ester and it has been one of the most valuable pharmacological tools in the study of carcinogenesis due to their effects as tumor promoters. TPA mediates PKC activation by binding competitively with DAG to the C1 domain causing an open conformation of PKC allowing for substrate-binding (27). Some caution should be made when using TPA for studying PKC effects, since other proteins than PKCs also are sensitive to this compound (12). Prolonged exposure to TPA and other phorbol esters leads to dephosphorylation and eventual downregulation of activated PKC by ubiquitination and proteasomal degradation (81,82).

PKC Inhibitors
The lack of selectivity among PKC inhibitors due to high similarity between the kinase domains of the different PKC isoforms has resulted in difficulties when specific PKC isoforms need to be inhibited and has also prohibited the use of inhibitors as therapeutic agents (30). Most has to be removed from the C4 domain (9). This occurs when the C1 domain binds DAG/phorbol esters and the interaction also stabilizes an open conformation of the PKC molecule making the catalytic domain accessible for substrate binding and phosphorylation (4,62). In the absence of C1 ligands no selectivity for negatively charged head groups in acidic lipids can be observed (64). Binding of the C2 domain to phosphatidylserine does not in itself cause activation or pseudosubstrate release.

Other views about the precise pathway by which PKC becomes activated includes one that suggests that activation of classical PKCs is initiated by Ca\(^{2+}\)-binding, which increases the affinity for phosphatidylserine and followed by binding to DAG at the plasma membrane (65). A third model speaks for an independent relationship between the C1 and C2 domains, targeting the membrane independently of each other (66).

PKC Phosphorylation
The phosphorylation motifs have been shown to be involved in an intramolecular clamp with the N-terminal part of the kinase as well as in an intramolecular interaction with the C2 domain (59,79).

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inhibitors target the ATP-binding site and this site is highly conserved among the PKC isoforms. Most PKC inhibitors belong to the indolocarbazol and the staurosporine-derived bisindolylmaleimide classes. G69976 belongs to the first class inhibiting classical PKCs, whereas GF109203X (also called bisindolylmaleimide I) belongs to the second class inhibiting all PKCs as well as other protein kinases (7). Calphostin C is another PKC inhibitor that instead of targeting the ATP-binding site targets the PKC C1 domain and impairs DAG/phorbol ester binding (83).

Metabolites from the sphingolipid metabolism are inhibitory second messengers for PKC. Sphingosine interacts with the regulatory domain of PKC and inhibits its activity either by interfering with the PKC-binding to phosphatidyserine or DAG (84).

Since most PKC isoforms are activated and inhibited by similar mechanisms, there is a need to develop compounds that influence PKC function in an isoenzyme-specific way. One approach has been to identify molecules that PKC-specifically either disturb anchoring or translocation of PKC (85). This has been accomplished by designing peptides that interfere with protein-protein interactions and peptides derived from the C2 domain of PKCζ, PKCδ, and PKCa have been shown to specifically inhibit translocation and function of the PKC isoforms they derive from (86,87).

Kinase-inactive or regulatory domains of PKCs are commonly used as dominant-negative inhibitors, but the high sequence similarity among the different PKC isoforms and the little knowledge about the intracellular targets of the constructs makes the use of these inhibitors questionable (48).

The PKC Expression and Function
PKCα, β, γ, δ, ε, and ζ are quite ubiquitous expressed and can be found in most tissues, whereas PKCγ, η, and θ are more restricted in their inhibitors target the ATP-binding site and this site is highly conserved among the PKC isoforms. Most PKC inhibitors belong to the indolocarbazol and the staurosporine-derived bisindolylmaleimide classes. G69976 belongs to the first class inhibiting classical PKCs, whereas GF109203X (also called bisindolylmaleimide I) belongs to the second class inhibiting all PKCs as well as other protein kinases (7). Calphostin C is another PKC inhibitor that instead of targeting the ATP-binding site targets the PKC C1 domain and impairs DAG/phorbol ester binding (83).

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expression (88-90). Of the PKC isoforms, PKCo is believed to be the most ubiquitously expressed PKC isoform (91). PKCs are involved in many normal cellular processes such as proliferation, differentiation, survival, and motility (Figure 4) (92-94). However, the PKC family has also been coupled to many other processes like cancer, pain sensation, diabetes, heart attack, stroke, heart failure, angiogenesis and the immune response (95).

PKC function depends on cell type and different subcellular localization of both PKC and its substrates (8,61,96,97). One PKC isoform can have one effect in one cell and another effect in another cell. In addition, the PKCs often have opposite effects to each other, complicating the analysis when trying to map a specific function to a specific isoform. Several approaches have been made to elucidate the roles of individual PKC domains in regulating PKC-specific functions. Construction of chimera proteins has helped to elucidate some unique PKC-specific domain functions. For example, has the regulatory domain of PKCα and PKCδ inhibits cell growth (98-100). A wide variety of PKC-interacting proteins with diverse functions have been identified illustrating the plethora of processes in which PKC is involved. Proteins, lipids, and second messengers have all been identified to bind different domains of PKC and some of the interacting proteins are PKC specific and PKC substrates, others are not. PKCα interact with many cytoskeletal proteins and many having positive influence on actin assembly and βI integrin expression (117,118). PKCα is also involved in activating the stress fiber-activating protein RhoA through binding to syndecan-4.

PKC Interactions

How can a protein that is a member of a group of proteins that share so many similarities, mediate unique cellular functions? The answer is by interacting with other proteins and these proteins regulate localization and function of PKC isoforms by several means (Figure 5). Some target PKCs to their proper cellular localizations in response to specific receptor-mediated activating signals. Some bring PKCs close to their substrates preventing inappropriate phosphorylations and others transport PKCs between different subcellular compartments. PKC-interacting proteins also help to integrate signals from different signaling pathways (96,114). Two categories of proteins function to bring PKCs close to their substrates (85). These are collectively called STICKs (substrates that interact with C-kinase) and RACKs (receptors for activated C-kinase). STICKs are phospholipid-binding proteins that are located in the interface between membranes and cytoskeletal structures and found in several different subcellular compartments. Phosphorylation of STICKs by PKC leads to a functional change of the STICKs, affecting cell morphology, adhesion, and cell spreading. STICKs bind transiently in vivo to the regulatory domain of PKCs and they are, at least in vitro, not isozyme-specific (96). RACKs selectively bind to a specific sequence in the regulatory domain. They were the first proteins identified as PKC-anchors and they function to anchor translocated active PKC to specific membranes but are not substrates (115). The C2 domain of PKC has been shown to contain a 6-10 amino acid long sequence identical to the sequence found in the PKC-interacting RACK and has therefore been named pseudo-RACK (30,87). Binding to RACK enhances the catalytic activity of PKC by inhibiting the intramolecular interaction between the pseudo-RACK and the RACK-binding site stabilizing the active conformation of PKC. The first PKC-selective RACK to be cloned was the PKCβII-selective RACK1 (116). RCKs (receptors for inactive C-kinase) are additional PKC-interacting proteins that are not PKC substrates (85). A wide variety of PKC-interacting proteins with diverse functions have been identified illustrating the plethora of processes in which PKC is involved. Proteins, lipids, and second messengers have all been identified to bind different domains of PKC and some of the interacting proteins are PKC specific and PKC substrates, others are not. PKCα interact with many cytoskeletal proteins and many having positive influence on actin assembly and βI integrin expression (117,118). PKCα is also involved in activating the stress fiber-activating protein RhoA through binding to syndecan-4.
PKCζ specifically binds to tubulin through its pseudosubstrate and the interaction is important for PKCζ function (132). Many PKC isoforms bind the conserved, ubiquitously expressed 14-3-3 family of scaffold proteins but so far the importance of these interactions in PKC signaling is not fully understood. There are indications of the 14-3-3 proteins both having PKC activity inhibiting as well as enhancing effects (133,134). PKCγ, α, θ, and μ have all been found to bind 14-3-3 proteins via their C1 domains. Some members of the 14-3-3 family bind selectively to specific PKCs, others show no PKC selectivity (135-138).

Other proteins have also been found to bind PKCs unselectively, such as the Bruton tyrosine kinase (Btk) that interacts with the C1 domain as well as the catalytic domain in several PKC isoforms (139,140).

PKC-Deficient Mouse Models

PKC-knockout mice have been established in an attempt to understand individual functions of PKC. However, a problem with studying PKC-knockout mice is the question of functional redundancy between the different PKCs. In addition, most studies on PKC deficiency have been done in specific settings like the effects on the immune system and in colorectal cancer (141,142). Different effects on the immune system and in colorectal cancer have so far been discovered in PKCα-deficient mice (145). No adverse phenotype has been found in PKCα-deficient mice compared to bacterial infections and increasing mortality as PKCα-deficient mice have impaired T-cell activation and proliferation whereas PKCβ-deficient mice have impaired T-cell activation (143,144). PKCε knockout mice show impaired macrophage function leading to lowered response to bacterial infections and increasing mortality as a consequence (145). No adverse phenotype has so far been discovered in PKCε-deficient mice (91). The roles of PKCs have been investigated in colorectal cancer by crossing PKC-deficient mice with mice prone to develop colorectal cancer. In a more cancer susceptible environment, PKCα deficiency enhances cancer progression by upregulating EGFR signaling, whereas PKCζ does not further potentiate the cancer phenotype. This is probably due to compensatory properties of PKCζ/θ. PKCζ/θ knockouts on the other hand are embryonic lethal (141).

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PKCs in Cancer

Dysregulation of PKC signaling may be one factor leading to uncontrolled cell proliferation and neoplastic transformation (7). Several PKC isoforms have been shown to mediate pathways important for tumorigenesis. Cell invasion has been shown to be mediated through PKCα, βI and II, or ε depending on tumor being investigated (101-103). PKCβII has been shown to induce hyperproliferation in colonic epithelium (104). PKCα and PKCε have in addition to tumor cell invasion also been implicated in regulating tumor growth and metastasis (105-109). However, targeting PKCs in cancer is quite complex, since the family consists of many members who could and probably have opposing effects and many PKCs have different functions in different cell types (107,110,111). For example, PKCε has been shown to be both pro-survival as well as pro-apoptotic (112,113). It would be ideal if a specific isoform in a certain cancer type could be targeted to inhibit its pro-tumorigenic properties and limit cancer spread (91).
The Neuronal Cell: Morphology and Pathogenesis

The nervous system consists of the central and peripheral nervous system (CNS and PNS). CNS includes the brain and spinal cord, whereas PNS refers to the somatic and autonomic nervous system. The somatic nervous system includes sensory and motor neurons. The autonomic nervous system, which control involuntary functions, can be further divided into a sympathetic and a parasympathetic part. During the development of the central and peripheral nervous systems many neurons undergo apoptosis and they also grow, remove and add neuronal processes and synapses all through life as part of a learning process (146). In contrast to many other cells, neurons are supposed to survive for the entire lifetime of the organism.

Morphology and Development

One of the hallmarks of a mature, terminally differentiated neuron is the cell shape (Figure 6). It consists typically of a cell body with unusually long cytoplasmic processes where the longest is called the axon. In comparison, the immature neuron called neuroblast is small and round without processes and markers for neural differentiation. During the development of the nervous system the neurons have to migrate to their proper locations, differentiate, polarize, extend and their axons and dendrites have to be guided into right regions and form synapses with appropriate targets (147,148). All of these processes depend on the neuron’s ability to respond to extra- and intracellular cues. The axon has to travel a long way, in humans as far as up to one meter, to synapse with their target cells and the dendrites have to undergo extensive growth and branching. The Notch signalling pathway has been shown to regulate dendritic growth and branching (149). At the tip of neuronal processes a specialized structure called the growth cone leads and directs the growth of the processes. The growth cone has an amoeboid movement due to actin assembly and disassembly of lamellipodia and filopodia that senses environmental cues (146,148). The lamellipodia and filopodia located in the right direction are stabilized by increasing cell adhesion and cytoskeletal stability (150). The axons and dendrites, important for receiving and transmitting electrical signals, are two distinct parts of the neuron and they differ in their protein and organelle composition. The axons are long and thin and of the same width, whereas the dendrites are short, thinner at their tips and heavily branched. Axons contain neurotransmitters in synaptic vesicles that they release at axon terminals upon stimulation, whereas dendrites contain receptors for released neurotransmitters (147).

The Role of the Cytoskeleton

Three distinct intracellular filaments constitute the cytoskeleton including the actin microfilaments (MFs), microtubules (MTs), and intermediate filaments (IFs). Both the actin filaments and the microtubules are essential for the cell and both are arranged so that the subunits give the filaments directionality. MFs are important for maintaining cell shape, structure, motility and transducing signals in signaling pathways, whereas MTs are involved in mitosis, motility, and intracellular transport (159,160). Much research has focused on the importance of microfilaments and microtubules during neuronal development (161). Actin microfilaments are expressed at the edges of growth cones whereas microtubules mainly are localized to the central parts of growth cones (162). IFs constitute together with MTs the principal filamentous network in the axon and other neuronal processes.

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The nervous system consists of the central and peripheral nervous system (CNS and PNS). CNS includes the brain and spinal cord, whereas PNS refers to the somatic and autonomic nervous system. The somatic nervous system includes sensory and motor neurons. The autonomic nervous system, which control involuntary functions, can be further divided into a sympathetic and a parasympathetic part. During the development of the central and peripheral nervous systems many neurons undergo apoptosis and they also grow, remove and add neuronal processes and synapses all through life as part of a learning process (146). In contrast to many other cells, neurons are supposed to survive for the entire lifetime of the organism.

Morphology and Development

One of the hallmarks of a mature, terminally differentiated neuron is the cell shape (Figure 6). It consists typically of a cell body with unusually long cytoplasmic processes where the longest is called the axon. In comparison, the immature neuron called neuroblast is small and round without processes and markers for neural differentiation. During the development of the nervous system the neurons have to migrate to their proper locations, differentiate, polarize, extend and their axons and dendrites have to be guided into right regions and form synapses with appropriate targets (147,148). All of these processes depend on the neuron’s ability to respond to extra- and intracellular cues. The axon has to travel a long way, in humans as far as up to one meter, to synapse with their target cells and the dendrites have to undergo extensive growth and branching. The Notch signalling pathway has been shown to regulate dendritic growth and branching (149). At the tip of neuronal processes a specialized structure called the growth cone leads and directs the growth of the processes. The growth cone has an amoeboid movement due to actin assembly and disassembly of lamellipodia and filopodia that senses environmental cues (146,148). The lamellipodia and filopodia located in the right direction are stabilized by increasing cell adhesion and cytoskeletal stability (150). The axons and dendrites, important for receiving and transmitting electrical signals, are two distinct parts of the neuron and they differ in their protein and organelle composition. The axons are long and thin and of the same width, whereas the dendrites are short, thinner at their tips and heavily branched. Axons contain neurotransmitters in synaptic vesicles that they release at axon terminals upon stimulation, whereas dendrites contain receptors for released neurotransmitters (147).

The Role of the Cytoskeleton

Three distinct intracellular filaments constitute the cytoskeleton including the actin microfilaments (MFs), microtubules (MTs), and intermediate filaments (IFs). Both the actin filaments and the microtubules are essential for the cell and both are arranged so that the subunits give the filaments directionality. MFs are important for maintaining cell shape, structure, motility and transducing signals in signaling pathways, whereas MTs are involved in mitosis, motility, and intracellular transport (159,160). Much research has focused on the importance of microfilaments and microtubules during neuronal development (161). Actin microfilaments are expressed at the edges of growth cones whereas microtubules mainly are localized to the central parts of growth cones (162). IFs constitute together with MTs the principal filamentous network in the axon and other neuronal processes.
The Intermediate Filaments

IFs have received their name because the filament size (10-12 nm) is intermediate in size of the other two filaments (159,172). The family of IFs is one of the largest gene families in the human genome and it consists of at least 67 genes (173). The IF proteins are divided into six groups (type I-VI) or four groups (A-D) depending on if the IFs are grouped according to genetic sequence or protein polymerization (174,175). The IF expression patterns are cell- and tissue-specific, which means that each cell type has a specific IF protein composition. IF expression patterns also correlate to a tissue’s differentiation state and the expression might therefore be under the regulation of different growth and differentiation factors (176). Unlike MFs and MTs, IF proteins are rod-shaped with a N-terminal globular head domain, a central conserved α-helical domain of fixed length (either ~310 or ~352 residues long) and a C-terminal nonhelical tail domain (177). Both the head and tail domains differ in length and primary structure between the different IF proteins and conduct the diversity observed in this family (174,178). They self-assemble into apolar filaments without any necessity of ATP or GTP and the filaments generally connect the plasma membrane with the nucleus (159,179). Whereas the central rod domain helps with the self-assembly, the N-terminal domain and the C-terminal domain, projecting from the filament core, are involved in IF regulation and protein-protein-interactions (174). Both ends contain regulatory phosphorylation sites. IFs have been found to be rather dynamic, but are still considered to be the most stable filament of the three cytoskeletal filaments. Many functions have been ascribed to the IFs, but they mainly function as supportive scaffolds. IF proteins are also important for various signaling pathways such as apoptotic signalling, mediating cytoskeletal crosstalk, and relaying signals from plasma membrane to nucleus, but also for processes like protein synthesis, mechanical stress protection, and cell migration (159,180-183). IFs also play crucial roles for the positioning of organelles, contributing to their shape and function, as well as for targeting proteins to right subcellular locations (184). During the neuronal development, IF proteins have different roles reflected by different localizations and expression patterns during different stages of development (163,164).

Immature, undifferentiated neuronal cells only express vimentin (165). Developing neuroblasts express IF proteins like nestin, α-internexin, and peripherin (166-168). Differentiation into mature neurons with axons triggers the expression of the neurofilament triplet proteins (NFTPs) (169). The NFTPs seem to be crucial for axon caliber in the mature axon (170). Mouse knockout experiments of individual IF genes do not have any severe effects on the development of the nervous system, axonal outgrowth proceeds as normal but some subsets of neurons are diminished (171).

Peripherin

Peripherin is a 58 kDa intermediate filament protein that was first identified in neuroblastoma and pheochromocytoma cell lines and initially only thought to be expressed in the neurons of the PNS (168,225,226). Later it has become apparent that neurons of the CNS have axonal projections towards peripheral structures, such as motor and sensory neurons, also express peripherin (227). Even some neurons having their entire cellbody and processes within the CNS, like neurons in the cerebellum, express peripherin (228). Peripherin belongs to the type III intermediate filament proteins together with GFAP, desmin and vimentin (177). Peripherin is first assembled into insoluble, nonfilamentous particles and then transported to the right location in the cell where it assembles with pre-existing filaments. Peripherin constitutes an IF network alone or together with the NFTPs (229,230). Three peripherin isoforms have been described in mice, having molecular weights of 56, 58 and 61 kDa, respectively (231). The 58 kDa variant is primarily expressed in vivo, whereas the 61 kDa variant is very toxic and causes peripherin aggregate formation and cell death when overexpressed in cultured neuronal cells (232). However, the splicing event that results in the 61 kDa variant is unlikely to occur in human (233). The biological function of peripherin is not yet fully understood. Peripherin mRNA and protein expressions increase after nerve injury, which might reflect a role in neuron regeneration (234).
Peripherin has its highest expression during early neuronal development. One potential role for peripherin during neuronal development might be in axonal guidance and outgrowth, since peripherin is expressed in neurons projecting their axons outside the CNS and studies performed in Xenopus embryos show peripherin being expressed in the distal region of the extending axon (164,230,239,241). In humans, all sensory neurons express peripherin and the majority of these possess thin unmyelinated axons and in the peripherin null mice fewer unmyelinated sensory axons are observed (245). Peripherin is also suggested to have a role in neuronal branching and branch stabilization, since a disruption of the peripherin network severely affects the axon sprouting capacity of affected neurons (240). Stimulation of rat pheochromocytoma PC12 cells, a cell line with high peripherin expression and widely used for studies on peripheral neurons, with NGF induces differentiation with neurite outgrowth and increased peripherin protein expression as a consequence (242). The importance of peripherin is further confirmed by the observation that downregulation of peripherin impairs processes like neurite initiation, extension, and maintenance in PC12 cells (243). However, there is contradictory work stating that knockdown of peripherin expression has no effect on NGF-induced neurite outgrowth in PC12 cells (244). In addition, peripherin knockout mice do not display any disruptions of processes like development, survival, and reproduction and seems to be indispensable for axogenesis. The pro-inflammatory cytokine tumor necrosis factor (TNF-α) as well as other inflammatory cytokines like interleukin-6 (IL-6) and leukemia inhibitory factor have been shown to upregulate peripherin expression (246-249). Exposure to ischemia induces peripherin expression in otherwise peripherin silent neurons (250).

Peripherin has been shown to be mainly phosphorylated on serine/threonine residues in its N-terminal half, like other class III IF proteins (251-253). However, there is evidence for tyrosine phosphorylation in the C-terminal of peripherin as well (251). TPA has been shown to increase peripherin phosphorylation and vimentin, a close homologue to peripherin, has been shown to be phosphorylated by PKC (242,254,255). Phosphorylation of vimentin leads to disassembly of the IF, whereas dephosphorylation allows for spontaneous self-assembly (159). However, peripherin is also phosphorylated during NGF-stimulation and this phosphorylation is independent of PKC activation (242). Peripherin was recently found to be a novel Akt substrate in neurons and the phosphorylation site, a serine residue in the head domain, was identified (235). How phosphorylation exactly regulates peripherin function is still not known. A member of the 14-3-3 family has been shown to bind the IF protein GFAP in a phosphorylation-dependent manner, speaking for phosphorylation creating new binding-sites for proteins important for IF dynamics (256).

Peripherin has been found in the majority of IF inclusion bodies seen in motor neurons of amyotrophic lateral sclerosis (ALS) patients, but also in other neurodegenerative disorders like Parkinson’s disease (238,257,258). Whereas peripherin deficiency does not lead to a severe phenotype, overexpression of peripherin in mice leads to a pathogenesis similar to that of ALS with the formation of peripherin inclusions and ultimately death of spinal motor neurons (259,260). The peripherin aggregates also seem to work in synergy with pro-inflammatory cytokines like the TNF-α to induce neuronal cell death (248). There might be an inverse relationship between the NFTPs and peripherin, since co-overexpression of the neurofilament protein NF-H prevents motor neuron loss seen in peripherin-overexpressing mice (261). In addition to neurodegenerative diseases, mutations of the IF genes have been found to be the cause of at least 30 different disorders like premature ageing, severe tissue fragility, and myopathies (262,263). Peripherin has also been found to be expressed in pancreatic islet β cells and suggested as a possible autoantigen in type 1 diabetes (264). Peripherin has also been found to be mainly phosphorylated on serine/threonine residues in its N-terminal half, like other class III IF proteins (251-253). However, there is evidence for tyrosine phosphorylation in the C-terminal of peripherin as well (251). TPA has been shown to increase peripherin phosphorylation and vimentin, a close homologue to peripherin, has been shown to be phosphorylated by PKC (242,254,255). Phosphorylation of vimentin leads to disassembly of the IF, whereas dephosphorylation allows for spontaneous self-assembly (159). However, peripherin is also phosphorylated during NGF-stimulation and this phosphorylation is independent of PKC activation (242). Peripherin was recently found to be a novel Akt substrate in neurons and the phosphorylation site, a serine residue in the head domain, was identified (235). How phosphorylation exactly regulates peripherin function is still not known. A member of the 14-3-3 family has been shown to bind the IF protein GFAP in a phosphorylation-dependent manner, speaking for phosphorylation creating new binding-sites for proteins important for IF dynamics (256).

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Regulators of Neuronal Processes

The RhoGTPases

The family of RhoGTPases has been shown to be involved in the neuronal developmental processes mentioned earlier (148,151). The RhoGTPases regulate dynamic changes of the actin cytoskeleton by transducing signals from extracellular stimuli (148). The RhoGTPases cycle between a GDP-bound inactive state and an active GTP-bound state. The guanine nucleotide exchange factors (GEFs) activate the RhoGTPases by facilitating the replacement of GDP to GTP, whereas the GTPase activating proteins (GAPs) inactivate the RhoGTPases by hydrolysing the GTP to GDP (152). The Rho family is further divided into three major groups where Rac1, Cdc42, and RhoA are the main members and the effects on the actin cytoskeleton differs between the members. In fibroblasts, Rac1 induces the formation of lamellipodia (ruffled membranes), Cdc42 induces filopodia (small protrusions), and RhoA induces stress fibers (filament bundles) and regulates focal adhesions (153-156). Rac1 and Cdc42 are positive regulators of neurite outgrowth and promote growth cone formation, whereas RhoA is a negative regulator that triggers growth cone collapse and neurite retraction in neuronal cells (157,158).

The PKCs

Neurites are the general name for processes (axons and dendrites) extending from neuronal cell bodies. Initiation, extension, and maintenance of neurites involve coordinated processes of different cytoskeletal proteins and the PKC family was early implicated to be involved in neurite outgrowth regulation (118,185). Treating neuroblastoma cell lines like SK-N-BE(2)/C with retinoic acid (RA) or stably TrkA-transfected SH-SY5Y with nerve growth factor (NGF) are two established differentiation model systems commonly used (186-188). The human neuroblastoma cell line SK-N-BE(2)/C originates from the SK-N-BE(2) cell line, which was isolated from the bone marrow of a 22-month-old male boy 1972. They are neuroblast-like, have small neurite-like processes, a nonfunctional, mutated p53, and a MYCN amplification (189). PKCα, βII, δ, and ε are highly expressed in human neuroblastoma cells (100). PKC-activating phorbol esters have been shown to stimulate differentiation and induce neurite outgrowth in several neuroblastoma cell lines (190,191). TPA also potentiates NGF-stimulated neurite outgrowth (191). Ethanol is another agent shown to increase NGF- or basic fibroblast growth factor (bFGF)-stimulated neurite outgrowth in PC12 cells by increasing the levels of PKCζ and PKCε (192,193). Induction of differentiation of LAN-5 neuroblastoma cells also leads to increased PKCζ levels compared to levels of PKCζ, γ, ζ, and η (194). However, there are also reports showing contradictory results where induced differentiation of either SK-N-BE(2)/C cells with RA or SH-SY5Y/TrkA cells with NGF does not lead to any increase in PKCζ protein expression (130,195). In addition, PKCα, as well as PKCε, has also been reported to be upregulated during neuronal differentiation as well as enriched in the growth cones (196). Both PKCζ as well as PKCε have been shown to be neurotogenic and the regulatory domains from the novel PKC isoforms δ, ε, and η all induce neurite outgrowth (130,195,197-201). Cellular processes have been induced in other cell types in addition to neuroblastoma cells, such as immortalized hippocampal neural precursor HiB5 cells and fibroblasts, and the ability to extend long processes is not something unique for neuronal cells but is rather a general process happening in many cell types (202-204).

PKC: However, full-length PKCζ is the only isoform that has been shown to induce neurite outgrowth and much research has focused on elucidating the mechanism whereby PKCζ mediates this effect. PKCζ is expressed in most tissues and cells, but with a higher expression in neuronal, hormonal, and immune cells and PKCε is the main PKC isoform being expressed in the central nervous system (205-207). Deleting the actin-binding motif in the full-length PKCζ abrogates the neurite-inducing ability of PKCζ but targeting PKC to the membrane restores the neurite inducing capacity. The importance of the link between actin and PKCζ is further supported by processes, a nonfunctional, mutated p53, and a MYCN amplification (189). PKCα, βII, δ, and ε are highly expressed in human neuroblastoma cells (100). PKC-activating phorbol esters have been shown to stimulate differentiation and induce neurite outgrowth in several neuroblastoma cell lines (190,191). TPA also potentiates NGF-stimulated neurite outgrowth (191). Ethanol is another agent shown to increase NGF- or basic fibroblast growth factor (bFGF)-stimulated neurite outgrowth in PC12 cells by increasing the levels of PKCζ and PKCε (192,193). Induction of differentiation of LAN-5 neuroblastoma cells also leads to increased PKCζ levels compared to levels of PKCζ, γ, ζ, and η (194). However, there are also reports showing contradictory results where induced differentiation of either SK-N-BE(2)/C cells with RA or SH-SY5Y/TrkA cells with NGF does not lead to any increase in PKCζ protein expression (130,195). In addition, PKCα, as well as PKCζ, has also been reported to be upregulated during neuronal differentiation as well as enriched in the growth cones (196). Both PKCζ as well as PKCε have been shown to be neurotogenic and the regulatory domains from the novel PKC isoforms δ, ε, and η all induce neurite outgrowth (130,195,197-201). Cellular processes have been induced in other cell types in addition to neuroblastoma cells, such as immortalized hippocampal neural precursor HiB5 cells and fibroblasts, and the ability to extend long processes is not something unique for neuronal cells but is rather a general process happening in many cell types (202-204).

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the observed increase in PKCε-actin-association during neuronal development and the fact that the neurite-inducing capacity seem to be dependent on Cdc42 (130,195,197,208). The neurite-inducing capacity of PKCε is dependent on extracellular stimuli, since removing serum from the medium diminishes its ability to induce neurites (130). In addition to being involved in neurite outgrowth-induction, PKCε has been shown to be involved in many other cellular processes such as proliferation, differentiation, gene expression, muscle contraction, mechanical force adaptation, metabolism, transport, exocytosis, endocytosis and other inflammatory, immune, and circular functions (see(205) and references therein).

The neurite outgrowth-inducing capacity of PKCε is independent of its catalytic activity and the regulatory domain of PKCε is even a more potent neurite-inducer than full-length PKCε (130). The region encompassing the pseudosubstrate, the two C1 domains, and parts of the V3 region (PSC1V3) in the regulatory domain is more neuritogenic than the regulatory domain. This construct can be used as a dominant-negative inhibitor of neurite outgrowth induced by differentiation agents (201). Furthermore, we have previously shown that the C1b domain together with twelve N-terminal and twenty C-terminal residues have the capacity to induce neurite outgrowth when targeted to the membrane and that a conserved motif N-terminally of the C1b domain, present in the novel PKCs and absent in PKCα, is necessary for neurite induction. Mutation of three residues in this motif (Phe237, Val239, Met241) abolishes the neurotrophic effect of PKCεPSC1V3. Amino acids located immediately N-terminally of the C1a domain has been shown to be important for membrane targeting and consequently neurite outgrowth induction (209). A way for PKCε to induce neurite outgrowth might be through changing its localization and/or conformation and this might be mediated through interactions with other proteins at the membrane that either anchor PKC to the membrane or induce a conformational change.

**Neuroblastoma**

During the neuronal development neural crest progenitor cells form a structure destined to be the spinal cord at the top of the neural tube. These cells later lay the ground for the entire PNS including the autonomic and peripheral ganglia and the adrenal gland (210). The adrenal glands produce many of the body’s vital hormones and they consist of an outer cortex and an inner medulla that are developmentally, functionally, and structurally very different from each other. The cortex releases several steroid hormones whereas the medulla, being a part of the autonomic nervous system, releases catecholamines, mainly adrenaline, and peptidohormones. Pathology of the glands is most often due to neoplasms and the most common tumor of the adrenal medulla originates from the chromaffin cells and is called pheochromocytoma. Other tumors arising from primitive neuroblasts of the sympathetic PNS include neuroblastoma and ganglioneuroma (211).

Neuroblastoma is together with medulloblastoma the most common primary tumor in childhood and it is alone the most common extracranial malignancy found in children, representing roughly 7-10% of all childhood cancers (210). Approximately 200 children between the years of 2000 and 2004 were diagnosed for neuroblastoma in the Nordic countries (Nordic Society of Pediatric Haematology and Oncology, 2007). Neuroblastoma is often diagnosed at around 18 months of age and can appear anywhere along the sympathetic PNS chain but roughly 65% of the tumors are found in the abdomen and in the adrenal medulla. The symptoms are linked to the location of the primary tumor and the most frequent symptom of neuroblastoma is abdominal pain due to abdominal distension (212,213). Unfortunately more than 50% of the patients have metastases at time of diagnosis and the most common locations of metastasis are regional lymph nodes, bone or bone marrow (211,212,214). Neuroblastomas detected in infants can regress spontaneously, even in the presence of metastases, or mature into benign ganglioneuroma and the outcome for these children is often better than for children older than 1 year of age (214). Patients with primary tumors during the neuronal development neural crest progenitor cells form a structure destined to be the spinal cord at the top of the neural tube. These cells later lay the ground for the entire PNS including the autonomic and peripheral ganglia and the adrenal gland (210). The adrenal glands produce many of the body’s vital hormones and they consist of an outer cortex and an inner medulla that are developmentally, functionally, and structurally very different from each other. The cortex releases several steroid hormones whereas the medulla, being a part of the autonomic nervous system, releases catecholamines, mainly adrenaline, and peptidohormones. Pathology of the glands is most often due to neoplasms and the most common tumor of the adrenal medulla originates from the chromaffin cells and is called pheochromocytoma. Other tumors arising from primitive neuroblasts of the sympathetic PNS include neuroblastoma and ganglioneuroma (211).

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located in the adrenal gland often do worse than patients with tumors arising at other sites (214).

Genetics
Most neuroblastomas are undifferentiated tumors and they are thought to develop due to disruption of the normal neural development; however, the exact mechanism behind this is not established. Searching for prenatal and/or postnatal environmental factors being responsible for neuroblastoma has so far been inconclusive. Most neuroblastomas occur spontaneously but there are cases of familial neuroblastoma and these are often diagnosed at an earlier age (215,216). Mutations in the ALK gene encoding a cell-surface kinase have just been discovered as the underlying cause to most familial neuroblastomas (217). About a third of the neuroblastoma tumors show amplification of the MYCN gene and this is the major marker for aggressive neuroblastoma correlating with poor prognosis (213,218). The transcription factor N-Myc is an important regulator of proliferation and differentiation of the developing peripheral neural crest and an inability of the early progenitor cells to exit from the proliferative phase could be a cause of tumor formation. High Myc pathway activation, not necessary due to MYCN gene amplification, has also been shown to correlate with poor prognosis (219). The TP53 gene, encoding the p53 protein, is often found mutated in cancers and an inactivation of p53 function contributes to malignant transformation. However, mutations in this gene are rarely found in primary neuroblastomas (220). High expression of the pro-survival nerve growth factor receptor TrkA is a favorable indicator of neuroblastoma (214). Aberrant Notch signaling has also been implicated in neuroblastoma (221). Many chromosomal aberrations are connected to neuroblastoma and unbalanced gain of distal 17q is the most common genetic abnormality found and deletion of 1p is found in 35% of primary tumors (222).

Treatment
To be able to offer the best possible treatment to children with neuroblastoma, the tumors are classified depending on tumor stage, age of patient at diagnosis, and site of primary tumor (214). The most commonly used staging system is called the International Neuroblastoma Staging System (INSS) and this is based on clinical, radiographic, and surgical evaluations of children with neuroblastoma (223). The use of a common staging system facilitates comparisons of treatments internationally. Treatment of childhood cancer is aggravated by the fact that childhood tumors differ from adult cancers since transformed normal cells are the source for proliferating tumor cells in adults whereas tumor cells in children originate from cells that already are rapidly dividing (210). Treatment of neuroblastoma begins with surgery and if needed continues with chemotherapy, and radiotherapy (224). During surgery, diagnosis is established, tissue samples are taken for further biological studies, the tumor is staged, and the entire tumor is excised without damaging adjacent tissue. Neuroblastomas often acquire drug resistance against drugs employed in therapy (189).

Amyotrophic Lateral Sclerosis
ALS, or Lou-Gehrig’s disease, is a rapid, progressive neurodegenerative disease where upper and lower motor neurons of the central nervous system degenerate (265). Examples of other neurological disorders where neuronal death underlies the symptoms are Alzheimer’s, Parkinson’s and Huntington’s diseases, and stroke. Motor neurons affected in ALS include the motor neurons of the cerebral cortex (upper motor neurons), brain stem and spinal cord (lower motor neurons) (266,267). It is one of the most common neurodegenerative diseases with adult onset, having an incidence of one-two per 100 000 in the population (268). The loss of motor neurons leads to atrophy of skeletal muscles and progressive paralyses of all extremities and respiratory and sip musculature. Only 25 % of the ALS patients live beyond five years after diagnosis and most patients die due to respiratory failure (269,270). Men and women are almost equally affected with some overrepresentation among men and, unfortunately, the number of people who succumb to the disease is increasing (271). Usually the disorder occurs sporadically with unknown origin, but approximately 10 % of the cases are familial. However, because there are no clinical or pathological differences between located in the adrenal gland often do worse than patients with tumors arising at other sites (214).

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Treatment
To be able to offer the best possible treatment to children with neuroblastoma, the tumors are classified depending on tumor stage, age of patient at diagnosis, and site of primary tumor (214). The most commonly used staging system is called the International Neuroblastoma Staging System (INSS) and this is based on clinical, radiographic, and surgical evaluations of children with neuroblastoma (223). The use of a common staging system facilitates comparisons of treatments internationally. Treatment of childhood cancer is aggravated by the fact that childhood tumors differ from adult cancers since transformed normal cells are the source for proliferating tumor cells in adults whereas tumor cells in children originate from cells that already are rapidly dividing (210). Treatment of neuroblastoma begins with surgery and if needed continues with chemotherapy, and radiotherapy (224). During surgery, diagnosis is established, tissue samples are taken for further biological studies, the tumor is staged, and the entire tumor is excised without damaging adjacent tissue. Neuroblastomas often acquire drug resistance against drugs employed in therapy (189).
the types, the cases cannot be distinguished from each other. However, sporadic ALS has a later onset than familial ALS (266,269). A typical pathological hallmark of ALS is the presence of inclusion bodies, also called spheroids, in the perikaryon and axon of affected motor neurons of both sporadic and familial ALS. These inclusions typically contain aggregated proteins like peripherin and NFTPs, the major IF protein in adult motor neurons (257,258,272,273). Abnormal IF accumulation is a pathological hallmark of many other neurodegenerative disorders besides ALS as dementia with Lewy bodies, Parkinson’s disease, and neuropathies like diabetic neuropathy (171). Many of the detected inclusions are positive for ubiquitin, a common feature for protein accumulations in almost all neurodegenerative disorders (272,274,275). IF inclusions can also be seen in liver and muscle disorders (276). However, whether or not the IF accumulations contribute to the ALS pathogenesis is still not known (277). There are several possible mechanisms whereby proteins accumulate such as deregulation of IF protein synthesis, abnormal phosphorylation, proteolysis, and other protein modifications (171).

Genetics
Little is known about what genetic defects contribute to ALS disease and so far no single gene has been shown to solely cause sporadic ALS (278). Several genes have been identified being mutated in ALS patients and these genes are often involved with intracellular trafficking and many of the mutations result in alternative splicing abnormalities (278). Due to the difficulty of investigating the ALS pathogenesis in humans, several mouse models have been created for investigating ALS origin and progression (245). One fifth of the familial ALS cases (or 5 % of the total ALS patients) have been found to be associated with dominantly inherited missense mutations in the gene coding for the free radical-scavenging enzyme copper/zinc superoxide dismutase-1 (SOD1) that exerts anti-oxidative and cytoprotective effects and is highest expressed in motor neurons (279-281). Mutations of the SOD1 gene are not restricted to familial ALS. They have also been found in patients with sporadic ALS (282). Mutated SOD1 is thought to result in ALS either by causing intracellular oxidative damages or by becoming misfolded or aggregated leading to toxicity (283,284). Different SOD1 variants are more prone to self-aggregate that wild-type protein in transgenic mice (295). Many explanations to how SOD1 aggregation could promote cytotoxicity have been proposed, ranging from disrupting of axonal transport (285), binding to regulators of apoptosis (286), inhibiting the proteasome (287), interfering with mitochondrial function and Ca²⁺ homeostasis (288,289), and being connected with glutamate excitotoxicity (290). Over 100 mutations in nearly 50 % of the amino acids in SOD1 have been found in ALS patients (291). Transgenic mice models where the SOD1 gene has been mutated in different ways all show pathological hallmarks of familial ALS (292,293). SOD1 deficient mice develop normally and do not show any traces of neurodegeneration, but they do not cope with axonal injury to the same extent as normal mice (294). This speaks for the toxic effects of mutant SOD1 not only being due to its diminished enzymatic activity. Both intracellular SOD1- and peripherin-containing aggregates appear in motor neurons of transgenic mice before any symptoms are visible, suggesting that the protein aggregations could be important for the pathogenesis (296). Other genes have been found to be mutated in cases of familial ALS and several candidate genes have been investigated for their involvement of sporadic ALS (271). ALS and dementia have been seen to occur in the same families and might therefore share a common genetic background (297).

Pathogenesis
So far, the complete picture of what causes the motor neuron death is not established. Much speaks for programmed cell death, apoptosis, being the process responsible for the observed cell death (298,299). The motor neuron degeneration observed in the human spinal cord can be divided into three consecutive morphological stages: cell death, followed by apoptosis and the so-called Nissl substance containing granular endoplasmic reticulum and ribosomes is dispersed; during the attritional the types, the cases cannot be distinguished from each other. However, sporadic ALS has a later onset than familial ALS (266,269). A typical pathological hallmark of ALS is the presence of inclusion bodies, also called spheroids, in the perikaryon and axon of affected motor neurons of both sporadic and familial ALS. These inclusions typically contain aggregated proteins like peripherin and NFTPs, the major IF protein in adult motor neurons (257,258,272,273). Abnormal IF accumulation is a pathological hallmark of many other neurodegenerative disorders besides ALS as dementia with Lewy bodies, Parkinson’s disease, and neuropathies like diabetic neuropathy (171). Many of the detected inclusions are positive for ubiquitin, a common feature for protein accumulations in almost all neurodegenerative disorders (272,274,275). IF inclusions can also be seen in liver and muscle disorders (276). However, whether or not the IF accumulations contribute to the ALS pathogenesis is still not known (277). There are several possible mechanisms whereby proteins accumulate such as deregulation of IF protein synthesis, abnormal phosphorylation, proteolysis, and other protein modifications (171).

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stage the cell body shrinks and the cytoplasm becomes homogeneous and the nucleus condenses; and, in the final apoptotic stage the cells round up exhibiting a condensed nucleus and contracted cytoplasm and only display one fifth of its original size. Apoptosis is characterized morphologically by plasma membrane blebbing with preservation of its integrity, cytoplasmic condensation, compaction of cytoplasmic organelles, chromat condensation, nuclear fragmentation, and finally the production of cell fragments surrounded by plasma membrane so called apoptotic bodies (270,301). Increased expression and activation of caspases-1 and -3 as well as apoptotic features support the apoptotic hypothesis (300,302,303). Many of the apoptotic changes are driven by caspases, a family of so far 14 inactive precursors that need to be proteolytically processed to generate active products. Caspases are activated by either the activation of the TNF family, the release of cytochrome C from the mitochondria to the cytosol, or by endoplasmatic reticulum (ER) stress (304). Nevertheless, it is very difficult to find conclusive evidence for apoptosis in ALS patients, since the apoptotic process is a rapid process of a couple of hours to a day and the ALS progress is relatively slow. The probability of finding many motor neurons undergoing apoptosis at any given time in a post-mortem specimen is quite low. Many sections must be examined to find any apoptotic indices in post-mortem tissue from ALS patients (304,305). Evidence for apoptosis in ALS patients have been found by identifying higher levels of DNA fragmentation by nick-end labeling and TUNEL staining in neuronal tissue samples compared to normal tissue (306,307). Pro-apoptotic proteins, like Bax, have also been shown to have higher expression than anti-apoptotic proteins, like Bcl-2, in motor neurons of ALS patients and this expression imbalance between pro- and anti-apoptotic proteins could explain the observed apoptotic process (300,306,308). However, there are reports not supporting the presence of apoptosis (309). For example, there are studies without positive TUNEL staining of neuronal tissue from ALS patients (310).

Apoptotic Mechanisms
There are several ways apoptosis can be induced in neurons (305). For example it has been shown that a lack of neurotrophic factors triggers apoptotic pathways during neuronal development (311). Another way is by overactivating glutamate receptors, a receptor present on most neurons in the CNS, leading to increased calcium influx. There are several reports showing an aberrant glutamate metabolism in ALS patients and elevated glutamate levels have been found in the cerebrospinal fluid of ALS patients (312,313). Oxidative stress can also induce apoptosis by the production of free radicals that damage nucleic acid, induce protein and lipid modifications. Evidence for oxidative injury has been found in ALS cases (265). Environmental toxins, like alcohol and cigarette smoking, are other examples of candidate ALS risk factors being investigated (314,315). However, no solid link between smoking and ALS has yet been established. Unfortunately there is not much research being done on apoptosis-inhibiting drugs and their effects on human degenerative diseases, due to low specificity and undesirable adverse effects of current anti-apoptotic substances (270,306). It has also been proposed a role for p53 in a motor neuron degeneration, since increased levels of p53 mRNA and protein has been associated with neuronal damage and an absent p53 protects neurons from toxic insults (299). Activation of the p53 pathway leads to a rapid increase of p53 mRNA and protein and this has been demonstrated in affected brain and spinal regions of ALS patients (316,317). However, p53 does not seem to be involved in all cases of ALS since it is death, since a crossbreed of SOD1-mutated mice and p53 deficient mice does not influence disease onset, progression or mortality induced by the SOD1 mutation (318).

Involvement of Peripherin
Abberant peripherin expression could be one of the factors that contribute to ALS pathogenesis. Unregulation of peripherin mRNA has been found in a familial ALS case (232). A recent study has identified a novel peripherin splice variant, designated Per28, being upregulated both on mRNA- and protein level in ALS (233). The 28 kDa splice isoform is present in motor neuron inclusion bodies. The same group also recently unveiled the need of another novel peripherin
isoform, 45 kDa, for normal network formation (319). In addition, an abnormal neurotoxic splice variant of peripherin, 61 kDa, has been identified in spheroids in ALS cases and has been shown to induce peripherin and neurofilament aggregation when expressed in motor neurons (232). Frameshift and point mutations in the PRPH gene coding for peripherin have been found in two ALS patients and these are both connected to peripherin aggregation (320,321). As mentioned earlier, peripherin expression can be increased by the inflammatory cytokine IL-6 and IL-6 levels have been found to be elevated in cerebrospinal fluid of an ALS patient (322).

Understanding the underlying mechanism of the neuronal cell death in ALS may provide new knowledge that can be used in the development of novel therapeutic techniques applied on neuronal protection in ALS patients. Furthermore, the development of effective neuroprotective strategies might be applicable on other neurodegenerative diseases like Alzheimer’s and Parkinson’s diseases.
mRNA Regulation

Post-transcriptional regulation is one way of regulating gene expression and cells recognize changes in growth conditions by changing the rate of messenger-RNA (mRNA) translation. Specific RNA-binding proteins are thought to regulate cytoplasmic mRNA processes like translatability, stability, degradation, and localization. Three RNA-binding proteins have been of specific interest to our research.

PABP

Pre-mRNAs undergo posttranscriptional modifications such as addition of a 5' 7-methyl-G cap and a 3' poly(A) tail. The poly(A) tail protects the mRNA from rapid degradation and it is involved in the processing and transportation of mRNA, as well as affecting translation efficiency. The poly(A) binding protein (PABP), discovered for more than 30 years ago, specifically binds to the 3' mRNA poly(A) tail and plays a role in translation initiation as well as in RNA stability, protecting its bound RNAs from nucleases attacks (323-325). Each poly(A) tail binds several PABPs (326). PABPs are divided into two groups, nuclear and cytoplasmic, based on their intracellular location but lately more members have been found and added to the family (327). PABPC1, also called PABP1 or simply PABP, is mainly cytoplasmic and ubiquitously expressed. The poly(A) tail-PABPC1 complex stimulates translation initiation by interacting with the translation initiation factor eIF4G, a translation initiation factor with a complex including other translation initiation factors, some bound to the 5' mRNA cap, ribosomal subunits, tRNAs, and accessory factors. Together PABPC1 and eIF4G form an 'end-to-end complex' or a 'closed loop' of the mRNA (324,328). PABPC1 also binds other eIF-interacting proteins further supporting the closed loop model (329). Translation initiation is the rate-limiting step during the translation process and at least 11 eIFs are needed for efficient translation. The PABPC1 sequence include four conserved, non-identical RNA-binding domains called RNA recognition motifs (RRMs) arranged in tandem as well as a less conserved C-terminal domain that does not bind RNA (324). The role of PABPC1 in mRNA stability is less understood than its role in translation, but there are reports claiming that PABPC1 influences the decapping pathway as well as 3'-5' exosome-degradation (330).

G3BP

The G3BP family of proteins is highly conserved between species and was originally identified in a screen for proteins binding to the SH3 domain of the RasGAP protein (331,332). RasGAP is a member of the Ras signaling pathway and the G3BPs have been shown to have a positive effect on cell proliferation and survival by affecting the cell cycle (331,333,334). G3BPs also affect cell growth by interacting with the cyclin-dependent kinase Cdk-7 and G3BP1 knocked out cells have reduced proliferation rate compared to control cells (335,336). The G3BP family consists of two highly homologous members, G3BP1 and G3BP2, encoded by genes located on separate chromosomes. G3BP2 additionally exist in two splice variants called G3BP2a and G3BP2b (337). The G3BP isoforms have a tissue-specific expression pattern and G3BP2 is the only isoform expressed in brain and muscle (337). The G3BPs are important during embryogenesis and a G3BP knockout is embryonic lethal, most likely due to neuronal cell death (336). The proteins all contain a N-terminal NTF2-like domain, a small SH3 domain binding sequence, an acid and proline rich central region and two different RNA-binding domains termed RRM and RGG in the C-terminal (338,339). What functions can be attributed the G3BPs and whether they have overlapping or complementary functions are not completely understood. In addition to Ras signaling, the G3BPs have been shown to be involved in many other signaling pathways. They influence NF-κB signaling by sequestering the NF-κB-inhibiting factor in the cytoplasm (340), p53 signaling by directly binding to p53 and its key regulator MDM2 and stabilizing both proteins (341), as well as HER2 signaling where increased HER2 signaling induces G3BP1 mRNA and protein expression in breast cancer cells (342). They are also thought to regulate RNA metabolism, mRNA Regulation

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but so far not many specific mRNA targets have been identified despite many efforts (336). They have been shown to bind specifically to c-Myc mRNA and to regulate its turnover by cleaving the 3’ untranslated region using an internal phosphorylation-dependent endonuclease activity (343,344). G3BPs also interact with ubiquitin specific proteases, but whether the interaction has an activating or inhibiting effect has not yet been established (345). G3BPs have also been linked to cancer where G3BP1 has been found to be overexpressed in multiple tumour tissues and correlate to aggressiveness of esophageal squamous carcinoma and G3BP2 has been found to be overexpressed in breast cancer tissue (333,338,346). G3BP1 has also been shown to induce specific RNA granules called stress granules (SGs) when overexpressed and the role of the G3BPs in the SGs might be to determine the fate of mRNAs during stress (347,348).

**IGF2BP**

The insulin-like growth factor-II mRNA-binding protein (IGF2BP or IMP) family consists of three highly homologous proteins that exhibit high affinity and multiple attachment sites for the mRNA encoding the growth factor IGF-II. IGF2BP1 was initially found to be a translation repressor of IGF-II mRNA during embryogenesis and IGF2BPs follow the expression pattern of IGF-II during development (349,350). During development IGF2BPs localize to cytoplasmic RNA-containing granules and these might constitute a specific entity of granules (351,352). IGF2BP1 expression is absent in most adult tissues except for testes and blood stem cells (353,354). IGF2BP3 was first identified as the protein KOC (KH domain containing protein overexpressed in cancer), which was overexpressed in pancreatic cancer cell lines and tissue (355). The IGF2BP family is suggested to have an important posttranscriptionally role by determining mRNA localization and stability as well as influencing translation initiation. IGF2BPs recognize and bind to specific mRNAs such as the c-Myc mRNA stabilizing and enhancing the mRNA half-life (356). Several reports show increased expression of IGF2BP1 in certain tumors (356-358). Downregulation of IGF2BP1 in breast cancer cells leads to downregulation of c-myc mRNA and protein, upregulation of IGF-II mRNA, and a decrease in cell proliferation (353). The IGF2BP chicken ortholog zipcode-binding protein (ZBP) has been shown to localize to SGs during stress (339).

**RNA Granules**

The formation of RNA containing granules so-called ribonucleoprotein (RNP) granules is a common cellular process and mRNA granules can be seen in germ cells as well as in neurons (360,361). The granules are especially evident in neuronal cells where RNA has to be transported over long distances to certain cytoplasmic compartments for local translation (362,363). Other examples of RNA-containing granules are the so-called processing bodies (PBs) (360). These regulate RNA metabolism by influencing RNA degradation, which occurs by two mechanisms; either by 3’-5’ exonucleases or by irreversible decapping. PBs are dynamic, cytoplasmic RNA granules containing translationally silenced mRNAs, decapping enzymes, and scaffold proteins (361,364-366). The size and number of PBs depend on the number of mRNAs undergoing decapping. mRNAs are constantly being screened for containment of nonsense codons through the nonsense-mediated mRNA decay (NMD) pathway and those mRNAs positive for these codons are targeted to the PBs (367). Even though PBs are mainly involved in mRNA degradation, there is emerging evidence that points to the fact that mRNAs present in the PBs can return to the cytoplasm and resume active translation (368). How many different RNA granules there are is not known and the identification of the compositions is given a lot of attention.

**Stress Granules**

Stress granules (SGs) are an additional example of mRNP granules. Stress, such as ultraviolet irradiation, heat shock, oxidative stress, ER stress, viral infection, hypoxia, and energy depletion, induces a cellular stress-response ensuring cellular survival. This response mainly affects the translation machinery by turning off general translation of “housekeeping” transcripts to conserve energy and protein, upregulating the expression of IGF-II mRNA, and decreasing in cell proliferation (353). The IGF2BP chicken ortholog zipcode-binding protein (ZBP) has been shown to localize to SGs during stress (339).
for repair of stress-induced damages (369). This in turn leads to an accumulation of translationally silenced mRNAs, which aggregate into cytoplasmic SGs (360,370-372). Translation of stress-induced transcripts encoding heat shock proteins (HSPs) among others is enhanced and stress-sensitive heat shock transcription factors (HSFs) regulate the stress-induced transcription in a stress-stimuli dependent manner (373,374). HSPs are a highly conserved set of proteins functioning as molecular chaperones that allow cells to adapt to changes in their environment and increasing cell viability in lethal conditions. There are both constitutive and induced HSPs and apart from being involved in protein folding, transport, and degradation, they are also major regulators of apoptosis by binding proteins involved in apoptosis formation and by directly interacting with caspases inhibiting their activity (375,376). Several stress-sensing serine/threonine kinases that in response to different cellular stress pathways; concentrating components involved in translation and promoting the assembly of translation initiation complexes (378). Heat shock-induced cytoplasmic granules were first observed in tomato cells and later in mammalian cells (379,380). Under elevated temperatures the nuclear actin filaments condense, the granularity of the nucleoli is altered, the cytoskeleton is rearranged, and the Golgi apparatus is disrupted (381).

The SG Formation

Several stress-sensing serine/threonine kinases accountable for initiating the cellular stress response have been identified, each specific for a certain type of stress. Double-stranded RNA-dependent protein kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK/PEK), general control nonrepressed 2 (GCN2), and hemeregulated inhibitor (HRI) are four kinases that in response to different cellular stress pathways the translation initiation factor eIF2α, which is an important component of the iRNA-complex carrying the first methionine in protein synthesis (382-385). Phosphorylated eIF2α blocks translation and the resulting stalled ribosomal complex with attached mRNA are readily shuttled to SGs (Figure 7) (360,386,387). This process requires the RNA-binding proteins TIAR and TIAR, which act downstreams of eIF2α phosphorylation and rapidly aggregate upon stress (371,388). The TIAR proteins are prominent markers for SGs (387).

SG Markers

SGs can be distinguished from PBs by the presence of translation initiation factors and ribosomal subunits in SGs. In spite of that there are proteins specific for either SGs or PBs, there are many proteins present in both granules suggesting a link between PBs and SGs (360). Indeed, fusions between the two structures have been reported (347,389). No one had until recently managed to purify a SG, but the discovery of the TIA-1/TIAR proteins enabled the identification of many SG components (364). SGs have been found to consist of proteins like exonucleases, scaffold proteins, RNA-binding proteins regulating mRNA structure and function as well as proteins that do not have anything to do with RNA metabolism (360,377). PABPC1 and G3BP are two proteins specifically expressed in SGs (371). Many of the proteins present in both granules suggest a link between SGs and PBs, but the discovery of the TIA-1/TIAR proteins enabled the identification of many SG components (364). SGs have been found to consist of proteins like exonucleases, scaffold proteins, RNA-binding proteins regulating mRNA structure and function as well as proteins that do not have anything to do with RNA metabolism (360,377). PABPC1 and G3BP are two proteins specifically expressed in SGs (371).}

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proteins identified as SG components can function as SG nucleators and induce SG formation when overexpressed. Examples are survival motor neuron protein (390), TIA proteins (391), Fragile X Mental Retardation protein (392), G3BP (347,348), caprin-1, a G3BP1-binding protein (393), and cytoplasmic polyadenylation element-binding protein (389).

The SG Regulation

There is not much known how stress granule assembly and disassembly is regulated. It is known that both PB and SG formation is promoted by blocking polysomes with for example puromycin and that their dissociation is promoted by stabilizing polysomes with cycloheximide (364). This phenomenon implies that translation must be switched off for mRNA to be able to relocate to SGs and PBs and that polysomes and SGs are in equilibrium during stress (388). In addition, SGs are absent and cannot be induced in mitotic cells due to stabilization of polysomes (394).

Several signaling cascades are induced during cellular stress and several of these pathways affect the subcellular distribution of RNA-binding proteins. Activation of the p38 stress-signaling pathway results in phosphorylation, cytoplasmic accumulation and alternative splicing of its downstream targets (395). Grb7 constitutes a family of conserved cytoplasmic adaptor proteins that have been found in SGs. Grb7 couples cell surface receptors with downstream signaling pathways. Grb7 interacts with the focal adhesion kinase (FAK) and FAK-mediated phosphorylation of Grb7 causes Grb7-release from SGs and sequential SG disassembly (396-398). SGs are believed to be connected to apoptotic pathways based upon the fact that SGs have been found to sequester proteins involved in TNF-α signaling and SGs are negatively regulated by the apoptosis-inducing factor (AIF) (399,400). In addition, SG formation has been shown to be dependent on an intact MT network and independent of the actin network (401). An attempt to identify what cellular factors control and/or influence the formation of SGs and/or PBs was recently made by using cells expressing fluorescently tagged SG and PB markers. These cells were thereafter used to study the effect of downregulating different protein expressions on SG and PB formation after stress-induction (402,403). Approximately 100 factors were found to affect SG formation indicating the complexity of these granules. Furthermore, O-linked N-acetylglucosamine modifications were shown to be of importance for SG formation and blockage of this pathway led to decreased SG formation in response to stress (403). Except for being positive for O-linked N-acetylglucosamine SGs are also positive for ubiquitin speaking for posttranslational modifications systems being closely linked to SGs (403,404).

Proteins identified as SG components can function as SG nucleators and induce SG formation when overexpressed. Examples are survival motor neuron protein (390), TIA proteins (391), Fragile X Mental Retardation protein (392), G3BP (347,348), caprin-1, a G3BP1-binding protein (393), and cytoplasmic polyadenylation element-binding protein (389).

The SG Regulation

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The present investigation

Aim and Introduction

The general aim of this thesis work has been to identify residues important for the neurite-inducing capacity of PKCε and to investigate by what mechanism PKCε mediates this property in neuroblastoma cells. A more detailed understanding of the role of PKCε in morphological differentiation of neuroblastoma cells will help us identify molecular targets that may improve treatment of neuroblastoma patients. Later in the project, the aim came to include elucidating the functional importance of the novel interaction between PKCε and peripherin, and its relevance for amyotrophic lateral sclerosis, and between PKCα and three RNA-binding proteins, and their involvement in stress granule regulation.

The major focus of the project has been to identify novel PKC-interacting proteins and establish the functional role of the interaction as well as with which specificity the interaction takes place. By determining the interacting motifs in respective proteins, novel therapeutic approaches can be taken to inhibit or facilitate the interaction. The use of selective inhibitors of protein-protein interactions might provide opportunities for subtle modification of cellular function that have different effects than inhibiting the kinase activity of the PKC isozymes. The SK-N-BE(2)C neuroblastoma cell line and the MDA-MB-231 breast carcinoma cell line have been used as a model systems.

The specific aims were:

► To identify specific amino acids in the C1b domain of PKCε important for its neurite-inducing effect
► To characterize the structural and functional importance of the novel interaction between peripherin and PKCε
► To characterize the structural and functional importance of the novel interactions between G3BP2, PABPC1, and IGF2BP3 and PKC
Results and Discussion

Ansn291 and some other residues at the base of the PKC εC1b domain are crucial for its neurite-inducing capacity (Paper I)

Inducing differentiation is one approach to slow the growth of neuroblastoma and our group has previously shown that PKCs induce neurite outgrowth, one feature characteristic for neuronal differentiation (201). The C1b domain has been found to be important for the neurotrophic property of PKCε and to highlight what residues in the PKCε C1b domain mediate the effect, we exchanged the εC1b domain for the αC1b domain in the PKCεPSC1V3 construct creating the PKCεPSC1a(αC1b)V3 construct. By doing this the PSC1V3 construct loses its neurite-inducing capacity, speaking for the existence of specific structures in the εC1b domain that are missing in the αC1b domain. We therefore aligned the C1b domains from PKCε and PKCα and found that out of 50 amino acids only 19 amino acids differed. By using the PKCεPSC1a(αC1b)V3 construct, the 19 residues in the εC1b domain were mutated one by one to the corresponding residue in the αC1b domain and tested for their importance for neurite-induction. Each new mutation was added to the former mutated PKCεPSC1a(αC1b)V3 construct. Mutating the PKCε residues Asp274, Ser290, and Leu291 into the PKCα residues Lys274, Pro290, and Asn291 led to a recovered ability of the PSC1V3 construct to induce neurites and introducing the single point mutation L291N was enough to give the εPSC1a(αC1b)V3 construct neurite inducing capacity in the presence of TPA although not as potently as wild-type. In addition, mutating Ansn291 in the PKCεPSC1V3 P290S construct into the corresponding PKCα residue led to a pronounced decrease of the number of cells with neurites both with and without TPA. We therefore used the model made of the PKCεC1b domain (27) to construct a three-dimensional model of the PKCεC1b domain and when studying the model it became evident that the three amino acids Lys274, Pro290, and Asn291 all are positioned in the lower region of the C1b domain opposite the DAG-phospholipol ester binding cleft. The previously described N-terminal motif also seems to be located close to the three important residues in the C1b domain when looking at the folded three-dimensional structure. In conclusion, Ansn291 located in the PKCεC1b domain was identified as an important amino acid for neurite-induction mediated by PKCε.

Peripherin, G3BP2, PABPC1, and IGF2BP3 were identified as novel PKC-interacting proteins (Paper II and III)

We next wanted to elucidate by which mechanism PKCε induces neurite outgrowth. One hypothesis is that PKCε mediates this effect by interacting with another protein at the plasma membrane or in close proximity of the membrane. Several screenings have been made and peptide libraries have been manufactured to find novel PKC interaction-partners (405,406). To screen for a novel interacting proteins that putatively could mediate the neurite-inducing effect of PKCε, we did an immunoprecipitation assay using the most potent neurite-inducing PKCε construct, the PKCεPSC1V3 domain, as bait. Mass spectrometry analysis resulted in the identification of several interesting interaction candidates (Table 1), including the class III intermediate filament peripherin and proteins being a part of the translation machinery such as the RNA-binding proteins PABPC1, G3BP2, and IGF2BP3. We next investigated peripherin and PKCε-mediated neurite outgrowth. However, so far we have not been able to demonstrate any involvement of peripherin, PABPC1, G3BP2, or IGF2BP3 in PKCε-mediated neurite outgrowth. The interactions between PKCε and the four proteins were confirmed by immunoprecipitation experiments with the endogenous proteins. The interaction between PKCε and peripherin was shown to be specific. However, it should be mentioned that some interaction could be observed between overexpressed PKCδ and 0 and endogenous peripherin, pointing towards the existence of a common structure shared between these PKC isoforms. PABPC1, G3BP2, and IGF2BP3 were shown to interact less specifically with PKCε. Interactions could be observed with

Results and Discussion

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endogenous PKCα and PKCβII as well. They all bound to overexpressed regulatory domain of PKCα, β, δ, and e. PABPC1 did not bind the regulatory domain of PKCβ. We next show that peripherin binds to the C1a domain of PKCε. Since we found a functional relationship between PKCε and the interacting RNA-binding proteins we went on with investigating what domain in PKCε mediated the interaction. These interaction experiments point to the C1a domain being the main domain mediating the interaction with PABPC1, G3BP2, and IGF2BP3. However, the C2 domain was also shown to interact with the PABPC1, G3BP2, and IGF2BP3, suggesting that PKCε has many interacting sites or that PKCε is a part of a bigger complex containing the RNA-binding proteins as well as others. The C1 domains have previously been found to mediate both isoforms-specific and -general protein-protein interactions (50,126,138,407). Neither of the novel interactions mentioned here seems to be dependent on the activation status of PKC, since the PKC activator TPA has no effect on the interactions. The functional importance of the interactions was thereafter investigated.

PKCε induces aggregation of peripherin when peripherin levels are increased leading to apoptosis (Paper II)

Overexpression of peripherin together with PKCε or in the presence of the PKC activator, TPA, leads to the formation of solid peripherin aggregates which in turn leads to death of the neuroblastoma cells. These novel findings can be of importance in understanding the mechanism behind the neurodegenerative disease ALS, where aggregates containing peripherin are essentially always seen in affected motor neurons (257). IF accumulations is a hallmark of both sporadic and familial ALS and understanding the role of the observed IF accumulations could reveal important information about some of the shared pathways between the two ALS types. It is still not known whether the IF accumulations actively participate in the neurodegenerative process, or if they just are a consequence of the pathogenesis. The PRPH gene has been found to be mutated in a few cases of familial ALS and peripherin expression has also been found to be enhanced in affected tissue (232,320,321). However, other genes have also been pointed out to be involved

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in the ALS pathogenesis. The SOD1 gene is one example and altering the peripherin expression levels in SOD1-mutated mice does not influence the degree of SOD1-induced pathogenesis in motor neurons. However, this only indicates that peripherin is not playing a central role in motor neuron degeneration caused by SOD1 mutations but might very well have an important role in other pathways leading to ALS (171).

Without any induction of aggregation, overexpression of peripherin does not in itself lead to cell death of neuroblastoma cells. However, it should be noted that an overactivation of PKC induced by high concentrations of TPA has been shown to cause neurodegeneration (408). However, the same phenomena could not be seen with lower TPA concentrations (408) and we have not observed any increased cell death during long-time exposure of TPA. Downregulating PKCε expression by using silencing RNA (siRNA) targeted towards PKCε leads to a reduction of cells with peripherin aggregates. Why this reduction is not complete, could either be due to the incomplete knockdown of PKCε or by the fact that peripherin also binds other novel PKCs which might have compensating functions. A decreased amount of cells containing peripherin aggregates can also be observed when inhibiting PKC activity or by co-expressing the peripherin-interacting domain of PKCε. By having a more detailed understanding of the function and molecular basis of the interaction between PKCε and peripherin we hope to find tools that prevent or interfere with pathological processes of importance for ALS such as peripherin aggregation.

**PKCε does not induce aggregation of vimentin, a close relative to peripherin, when vimentin levels are increased (Paper II)**

We have also investigated whether PKCε had the same effect on vimentin, a related type III IF protein (159), or if this effect was peripherin specific. Vimentin has previously been shown to interact with PKCε (409) and here we show that vimentin interacts with the same PKCε region as peripherin encompassing the pseudosubstrate, C1 domains and V3 domain. However, activation of PKC did not lead to aggregation of overexpressed vimentin. In addition, punctuate structures formed when exogenous vimentin mutants are expressed (409) did not contain peripherin and were not affected by exposure to TPA, indicating that these vimentin structures were distinct from the peripherin. Both we and others have shown that increasing the levels of peripherin enhances its tendency to aggregate (410). Much research has been done to investigate the self-assembling capacity of the IF proteins and disruption of this capacity often leads to the accumulation of the proteins in aggregates. We further investigated the role of the head respectively tail domain of peripherin in peripherin aggregation and we found that deleting either the head or the tail domain of peripherin both decrease the propensity of peripherin to aggregate (Figure 8). However, whereas the deletion of the tail domain completely abolishes the aggregating capacity in the presence of TPA, deleting the head domain only leads to a minor decrease of the amount of cells with peripherin aggregates after TPA treatment. The N-terminal head of peripherin has been shown to be essential for peripherin self-assembly and tailless peripherin protein can self-assemble into filament bundles (411,412). Deleting the C-terminal together with parts of the central rod domain of the IF proteins disrupts the self-assembling capacity and results in aggregate formation (413).

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None of the PKC-interacting proteins have so far been confirmed to PKC substrates (Paper II and III)

Many PKC-interacting proteins are PKC substrates. Since TPA had a potentiating and GF109203X had an inhibiting effect on peripherin aggregation, we hypothesized that PKCε-dependent phosphorylation of peripherin could be a determining factor for peripherin aggregation. However, we have so far not been able to demonstrate an increase of peripherin phosphorylation after PKC activation though this has been shown in PC12 cells (242). The dependence on PKC kinase activity may be explained by the presence of another protein promoting peripherin aggregation when phosphorylated by PKCε. We have so far not been able to demonstrate any increased phosphorylation status for PABPC1, G3BP2, or IGF2BP3 either after PKC activation. Phosphorylation of G3BP1 has been shown to influence both its internal RNase activity and its subcellular localization and one phosphorylation site has been found for casein kinase II (343, 344). Both PABPC1 and translation initiation factors are known phosphoproteins, where phosphorylation regulates the protein-protein-interactions as well as the protein-mRNA-interactions.

Hyperphosphorylated PABPC1 binds less efficiently to poly(A) mRNA and depending on the level of phosphorylation PABPC1 attracts different sets of initiation factors (414). However, how PABPC1 is phosphorylated is not known. The MKK-2/ERK pathway has been implicated to be one possible pathway regulating PABPC1 phosphorylation (415). Whether IGF2BP2 are regulated by phosphorylation remains to be studied. PKCε has been shown to, at least in vitro, phosphorylate factors involved in translation initiation as well as elongation (416). It would be interesting to continue investigating more of the proteins detected in our assay. For example, the eEF1α protein identified as a PKCε-interacting protein has in vitro been shown to be phosphorylated by PKCε (417).

G3BP2, PABPC1, and IGF2BP3 PKCε are crucial for stress granule formation (Paper III)

The three RNA-binding proteins were all found to relocate to stress granules induced by 44°C heat shock in SK-N-BE(2)C neuroblastoma cells and MDA-MB-231 breast carcinoma cells, as have been previously shown in other cell types (359, 364). Heat shock is a commonly used stress inducer and temperatures ranging from 42-45°C have been used to stress cells (359, 371). We have also induced SGs using the proteasome inhibitor MG-132 in SK-N-BE(2)C neuroblastoma cells and MDA-MB-231 breast carcinoma cells, consistent with prior reports (418). Both PABPC1 and G3BP2 were used as stress granule markers, since they have been shown to be specific for stress granules (364). However, the antibody used for G3BP2 does not distinguish between G3BP2a and G3BP2b variants. In spite of that, PABPC1, G3BP2, and IGF2BP3 were found in a co-immunoprecipitation assay using PKCε, to our surprise, PKCε was the predominant PKC isoform localizing to the stress granules during heat shock. However, G3BP2 has been previously shown to be a component of an mRNP also containing active PKCθII and RACK-1 and PKCθII has been shown to have a translation regulatory function by interacting directly to ribosomes and via associating with RACK1 (416, 419). The PKCs have been previously shown to be important stress sensors. Both PKCε and PKCγ have protective roles against decreased oxygen levels during stroke, neural and cardiac ischemia (420-422). PKCγ has been shown to be important during ER stress or amino acid deprivation, having a crucial role in the formation of autophagosomes that are formed during the cellular response (423). In addition to interacting with PKCs, we saw that each RNA-binding protein seems to bind to each other or at least exist in the same complex. IGF2BP1 and G3BP2 have previously been shown to associate RNA-dependently with G3BP1 and HnRNP A1, and other RNA-binding protein (424).

PKCε, G3BP2, and PABPC1 are crucial for stress granule formation (Paper III)

When we downregulated G3BP2, fewer cells with heat shock-induced SGs in neuroblastoma cells could be observed. In neuroblastoma cells, knockdown of either PABPC1 or IGF2BP3 had no affect on the SG forming ability. However, knockdown of both G3BP2 and PABPC1 in None of the PKC-interacting proteins have so far been confirmed to PKC substrates (Paper II and III)

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PKC has previously been shown to be involved in other aspects of mRNA regulation. For example, PKC has in several studies been shown to increase mRNA stability when activated (425-427). Neither PKC activation nor PKC inhibition has any affect on the assembly or disassembly of SGs and we, as well as others (364), have not observed that TPA treatment in itself induce the formation of SGs. The exact function of SGs is not well understood. SGs are thought to sequester specific mRNA under stress and in that way repressing translation of some mRNA and facilitating translation of other mRNA. However, cells that are unable to form SGs are still able to repress translation (404), speaking for SGs having additional roles. Several questions remain to be answered concerning whether SGs play any role in RNA silencing under normal physiological conditions and whether SGs induced by different stimuli contain the same or different components. Neither does one know how the formation of SGs affects cell vitality and protein synthesis. 

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Conclusions

- Residues at the base of the PKCε C1b domain, especially Asn291, are essential for the neurite-inducing capacity of PKCε.

- Peripherin was found as a novel PKCε-interacting protein and either increased PKCε-levels or PKC activation mediates peripherin aggregation. Induced peripherin aggregation also leads to increased cell death.

- The RNA-binding proteins G3BP2, PABPC1, and IGF2BP3 were found to be novel PKCα-interacting proteins that relocate to stress granules under stress. Knockdown of G3BP2, PABPC1 or PKCα block stress granule formation during cellular stress.

- Neither peripherin nor the RNA-binding proteins found in the PKCε-interaction assay affect PKCε-induced neurite outgrowth.


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First I want to thank Christer for always being there, for always helping with any scientific problems or questions no matter what, being super busy, away on a meeting, on vacation or at home. You have provided a challenging environment, stimulating me to always do my best and think creatively. You have allowed me to be as self-going as I have wanted to be at the same time as you have always showed your interest for everything I was doing. I couldn’t have wished for a better supervisor.

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If I have forgotten someone, please don’t be mad - I didn’t mean it!

I couldn’t have done any of this without my family! I want to thank my brother, Henning, for helping me nourish my other interests outside the scientific world and for always looking out for me. I especially want to thank “Mamma” and “Far” for always supporting me in everything I do, for always encouraging me to do my best, for always having the time to listen, and for all hugs that lift me up when I’m down. There are no words to describe how much you mean to me!

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