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Interplay Between Protein Kinase C Isoforms Alpha and Epsilon, Neurofibromin, and the Ras/MAPK Pathway in Neuroblastoma Differentiation

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1. Introduction

Neuroblastoma (NB) [1] is the most common extracranial tumor in childhood and accounts for nearly half of neoplasms diagnosed during infancy (Maris, 2010; Brodeur, 2003). A striking feature of these tumors has been their heterogeneous course, which ranges from spontaneous regression to inevitable progression and death (Brodeur, 2003). Current pharmacological approaches in the treatment of NBs include standard combination chemotherapy using dose-intensive cycles of carboplatin, etoposide, cyclophosphamide, and doxorubicin, with the addition of topoisomerase I inhibitors. For intermediate-risk NB, a high rate of survival among patients may still be achieved with significant reduction of doses and duration of chemotherapy (Baker et al., 2010). The retinoic acid analogue isotretinoin (13-cis-retinoic acid) is additionally used in high-risk NB patients with progressive or recurrent disease (Maris, 2010; Reynolds et al., 2003).

Animal cancer models have offered valuable preclinical testing systems for studying the impact of specific genes in the appearance and the progress of the disease as well as the efficacy of novel therapeutic regimes. Animal models of NB have been developed by subcutaneous inoculation (xenografting) of established human NB cell lines in immunocompromised mice; for instance, the cell line SK-N-BE2c was successfully used to develop an animal model and test the effects of imatinib (Meco et al., 2005). Yet, the major

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[1] Abbreviations used: ERK, extracellular signal-regulated kinase; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; DAG, diacylglycerol; TH, tyrosine hydroxylase; GAP43, growth-associated protein 43; NPY, neuropeptide Y; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; dBcAMP, dibutyryl cAMP; BrdU, 5-bromo-2-deoxyuridine.

drawback was the lack of metastases. Pseudometastatic models were next developed by tail-vein injections of human NB cell lines in athymic mice (Pastorino et al., 2010). A clinically relevant model should, however, reflect the characteristics of advanced NB in children, that is large adrenal gland tumors and multiple small metastatic lesions. Such models have been generated only in nude mice with murine NB cells (NXS2) or by allografting human NB cell lines (Pastorino et al., 2010). Spontaneous adrenal neuroblastomas may be developed, yet rarely, in experimental animals (Rice, 2004), while, neither carcinogen-induced nor genetically engineered animal NB models have been successfully generated thus far.

Therefore, the development of novel therapeutic strategies, including both new targets and models, is urgently needed. Progress is expected through elucidation of the key molecular pathways that drive NB proliferation, differentiation, or apoptosis. Clinical trial data have also suggested that induced differentiation may be an alternative therapeutic approach, and retinoic acid analogues have been introduced in the clinical practice (Reynolds & Lie, 2000; Brodeur, 2003). The clinical observation of spontaneous differentiation into benign ganglioneuromas has provided the basis for studying neuronal differentiation of NB cells in culture (Reynolds & Lie, 2000; Edsjo et al., 2003; Hahn et al., 2008), and such studies have provided invaluable mechanistic insight into the fundamental mechanisms of neuronal differentiation and neurotransmitter phenotype acquisition (Mangoura et al., 2006b; Edsjo et al., 2007). The agents, mostly used to study NB cell differentiation in culture are phorbol esters, membrane permeable non-hydrolyzable cAMP analogues, and the clinically relevant vitamin A metabolite retinoic acid, alone or in combination with specific neurotrophic and growth factors (Table 1). These agents target distinct proximal signalling pathways: phorbol esters activate novel and conventional protein kinase C (PKC) isoforms, cAMP analogues activate protein kinase A (PKA) and exchange proteins activated by cAMP (Epacs), while retinoic acid acts as a ligand for nuclear hormone receptors/transcription factors (RARs) (Table 1). Yet, a significant level of crosstalk amongst these agents has been demonstrated in the induction of NB differentiation. Activation of PKCs and the ensuing Ras/ERK signalling cascade have been highlighted as central modulators of NB differentiation, with novel (PKCε) and conventional (PKCα) PKC isoforms critically controlling the signalling output and dynamics of MAPKs (Griner & Kazanietz, 2007). The importance of the PKC/Ras/ERK pathway is further emphasized by recent studies showing that neurofibromin, a prominent tumor suppressor and a neuronal RasGAP protein (a) is a PKCα and PKCε substrate (Mangoura et al., 2006a) actively phosphorylated during phorbol ester-induced differentiation (Leondaritis et al., 2009) and (b) provides responsiveness to retinoic acid (Holzel et al., 2010). In these studies, the role of PKCα and PKCε may be viewed as differential and even opposing, with PKCε emerging as a crucial, neuronal differentiation-specific PKC isoform.

In this chapter, we introduce the basic aspects of the PKC/NF1/Ras/ERK pathway and its implications in neuronal differentiation, we discuss critical findings from studies with NB cells that highlight the importance of this pathway in NB differentiation, and present original experiments that further expand current knowledge. Finally, we propose that agents promoting NB differentiation via distinct primary targets may actually converge on establishing a balance between PKCα and PKCε activities that coordinates neurofibromin (NF1)-dependent Ras/ERK activation and NB differentiation.

2. PKC isoforms, NF1, and the Ras/ERK pathway in differentiation of neuroblastoma cells

2.1 The PKC/NF1/Ras/ERK pathway

2.1.1 The PKC family: Structure, regulation, and substrates

The PKC family of serine/threonine protein kinases consists of at least ten isoforms that are classified into three subgroups based on their structure and specific cofactor requirements. Conventional PKCs (cPKCs: α, βI, βII and γ) are activated by the second messengers Ca^{2+} and DAG, while novel PKCs (nPKCs: δ, ε, η and θ) are activated only by DAG. In contrast to conventional and novel, atypical PKCs (aPKCs: ζ and λ/ι) are insensitive to both Ca^{2+} and DAG, but responsive to the Par6–Cdc42 complex (Parker & Murray-Rust, 2004; Rosse et al., 2010). PKCs are considered hubs for the transduction of signals from G protein-coupled and tyrosine kinase receptors (Parker & Murray-Rust, 2004; Griner and Kazanietz 2007), having long been recognized as a link between receptor-dependent generation of DAG by phospholipases C and D, and the key event for enganging the ERK pathway, the activation of Ras and Raf (Mangoura and Dawson, 1993; Marais et al., 1998). Attesting to the widespread effects of PKC activation, numerous studies have investigated the involvement of all PKC isoforms in oncogenesis and cellular differentiation, proliferation, polarity, migration, apoptosis, and survival (Bosco et al., 2011; Rosse et al., 2010; Larsson, 2006).

PKCs are founding members of the AGC kinase group and share a common structure consisting of a conserved C-terminal kinase catalytic domain and a more divergent N-terminal regulatory region. The regulatory region contains C1 and C2-domains (cPKC and nPKCs) that recognize the second messengers DAG/phospholipids or Ca^{2+}/phospholipids, respectively, and a pseudosubstrate sequence that serves in autoinhibition by interacting with the substrate-binding pocket of the catalytic domain. Binding of second messengers or allosteric effectors on the C1/C2-domains of the regulatory region results in stabilized interaction with the plasma membrane and activation (Parker & Murray-Rust, 2004; Griner and Kazanietz 2007). PKCs are additionally regulated by specific phosphorylation events that "prime" the kinase for activation. As for all AGC kinases, PDK1 phosphorylates threonines (Thr) in the activation loop (Thr566 in PKCε). Residues in the turn motif (Thr710 in PKCε) and the C-terminal hydrophobic motif of c/nPKCs (Ser729 in PKCε) are often phosphorylated by the mTORC2 complex (Freeley et al., 2011). These phosphorylations critically depend on the occupation of the catalytic site by ATP, stabilize the active conformation of PKCs, and result in a fully primed kinase (Cameron et al., 2009). Additional auto- or in-trans phosphorylation events by other PKC isoforms may have more subtle effects, such as modulation of catalytic activity, protein stability, or binding to other proteins (Freeley et al., 2011). Specifically for PKCε, phosphorylation on Ser368 in the regulatory region by cPKCs suggests a high level of functional crosstalk within members of the PKC family (Durgan et al., 2008).

By the acute and reversible post-translational modification of phosphorylation, PKCs regulate the activity and subcellular localization of several of their protein substrates. PKCs phosphorylate an array of substrates that include transcription factors, receptors, ion channels, and cytoskeletal proteins (Mangoura and Dawson 1993; Mangoura, 1997; Parker & Murray-Rust, 2004; Larsson, 2006; Mangoura et al., 2006a). In many cases, however, proof of direct PKC phosphorylation of substrates in vivo has remained limited, with several molecules representing remote "PKC targets". PKCs also act as scaffols, as they interact with other proteins independently of their enzymatic properties as kinases (e.g. Saurin et al.,

2008; Valcova et al., 2007). Mechanistically and pharmacologically important PKC-binding proteins are the RACKs (receptors for activated C-kinases), which stabilize the interaction of activated PKCs with plasma membrane (Schechtman & Mochly-Rosen, 2001). Studies of the PKC-RACK interactions by Mochly-Rosen and co-workers, have shown that isoform-specific activators or inhibitors may be rationally designed, from unique 7-8 amino acid sequences in each PKC or in their respective RACKs (Budas et al., 2007; Csukai & Mochly-Rosen, 1999). As such, εV1-2 and ψεRACK peptides, specific inhibitor and activator peptides for PKCε, respectively, and analogous peptides for other PKCs have been used in numerous studies in culture and in vivo with great success (Asimaki and Mangoura, 2011; Asimaki et al., 2011).

2.1.2 The Ras/ERK pathway and modulation by PKCs

Ras GTPases, are key molecules for growth and neurotrophic factor signalling through the three kinase MAPK module, consisting of Rafs (MAPKKK), MEK1/2 (MAPKK), and ERK1/2 (MAPK) (Raman et al., 2007 and refs. therein). All Ras proteins, namely H-, N- and K-Ras, cycle between active GTP-bound and inactive GDP-bound states; activation of guanine nucleotide exchange factors (GEFs) after membrane receptor stimulation promotes the exchange of GDP for GTP and activation of Ras (Buday & Downward, 2008). The opposite event is controlled by GTPase-activating proteins (GAPs) that activate the intrinsic GTPase activity of Ras and lead to GTP hydrolysis and Ras inactivation (Scheffzek et al., 1998). Ras-GTP recruits Raf to the membrane and together with other kinases activates Raf kinase activity (Stokoe and McCormick, 1997). Subsequently, relay phosphorylations through MEKs result in phosphorylation and activation of ERK, which is considered as the terminal effector of the pathway (Raman et al., 2007). The Ras/ERK pathway controls various cellular processes such as proliferation, migration, and differentiation. A recent review by Katz et al., provides an overview of the Ras/ERK pathway impact on cellular differentiation and oncogenesis (Katz et al., 2007). Our focus is the PKC input on the regulation and dynamics of activation of Ras and ERK.

Mechanistically, PKC activation is necessary for the formation of Ras and activated Raf-1 complexes (Marais et al. 1998; Hamilton et al., 2001). PKCs may directly phosphorylate Raf (Ueda et al., 1996), RasGEFs (Ebinu et al. 1998; Roose et al., 2005; Zheng et al., 2005), and RasGAPs (Izawa et al., 1996; Mangoura et al., 2006a; Leondaritis et al., 2009), hence regulating the output of Ras/ERK signalling at multiple levels. PKCs on GEFs: Members of the GRP family of RasGEFs possess C1-domains that "recognize" DAG produced by phospholipases in the membrane upon membrane receptor stimulation, and through these interactions translocate to the membranes too (Ebinu et al. 1998). The GEF activity of RasGRP1,3 is greatly enhanced by concurrent phosphorylation by DAG-activated nPKCs, at least in T- and B-cells (Roose et al., 2005; Zheng et al., 2005). PKCs may also activate SOS1, the other major RasGEF, directly by phosphorylation (Rubio et al., 2006) or indirectly, by recruiting Grb2/SOS1 complexes via the Syk tyrosine kinase (Kawakami et al., 2003). PKCs on RasGAPs: Most of the cellular RasGAP activity is attributed to p120GAP and neurofibromin, and earlier studies have suggested a PKC-dependent inhibition of RasGAP activity in certain cell types (Downward et al., 1990). PKCs indirectly modulate the GAP activity of p120GAP via its interaction with RACK1 (Koehler & Moran, 2001), and neurofibromin, the main neuronal RasGAP, is directly regulated by PKC. Neurofibromin is phosphorylated by PKCs in vitro, particularly at the C-terminal domain (Izawa et al., 1996). Evidence for direct PKCα and PKCε-dependent neurofibromin phosphorylation was

documented later in cultured neurons, and neuroblastoma and glioma cell lines (Mangoura et al., 2006a). Moreover, phosphorylation of neurofibromin results in both its increased association with actin and enhancement of its GAP activity (Mangoura et al., 2006a). PKC-phosphorylation sites on neurofibromin are present in the N-terminal CSRD domain (Mangoura et al., 2006a) and in the C-terminal domain (Leondaritis et al., 2009). The residue Ser2808 in the C-tail of neurofibromin is indeed a PKC-specific site and its phosphorylation correlates well with enhanced signalling through the Ras/ERK pathway in TPA-treated SH-SY5Y cells (Leondaritis et al., 2009). Interestingly, studies with glioblastoma cell lines or tumors have indicated that PKCα may promote proteasome-dependent neurofibromin proteolysis, hence insufficient neurofibromin RasGAP activity to control Ras and accelerated cellular proliferation (McGillicuddy et al., 2009). Whether neurofibromin phosphorylation on Ser2808 (or other sites) is directly involved is, however, unknown. In sharp contrast, phosphorylation of neurofibromin in-non tumor cellular contexts has been correlated with increased stability of the protein, at least in melanocytes (Kaufmann et al., 1999) and neurons (Mangoura et al., 2006a). In conclusion, PKCs have the potential to directly impact on the activation state of Ras by modulating the activity of both GEFs and GAPs in a variety of ways. It should be also noted that in certain cell lines, PKC-dependent ERK activation may occur independently of Ras, since the dominant-negative form of Ras (RasS17N) that resists GEF-dependent activation does not inhibit TPA-induced ERK activation (Ueda et al., 1996; Rubio et al., 2008 and refs. therein). Overall, c/nPKCs like PKCα, PKCη, PKCδ and PKCε and aPKCs have been suggested to provide an activatory input on ERK pathway mostly at the level of Raf (Ueda et al., 1996; Schönwasser et al., 1998; Paruchuri et al., 2002).

2.1.3 NF1 and PKCε in neuronal differentiation

All major transduction molecules of the Ras/ERK pathway, namely Ras and Raf, are also potent inducers of differentiation and neuritic outgrowth in several neuronal cell lines (Wood et al., 1993; Olsson & Nanberg, 2001; Hynds et al., 2003). Moreover, it is the intensity and duration of Ras/ERK activation that determines the biological outcome. Seminal observations were first made in PC12 cells treated with EGF or NGF. Both growth factors activate the same Ras/ERK pathway, yet, transient ERK activation by EGF induces cell proliferation, whereas a sustained ERK activation by NGF induces neurite outgrowth and differentiation (Marshall, 1995 and refs. therein). The reasons for these fundamental differences are still debated (Santos et al., 2007; von Kriegsheim et al., 2009). At any rate, both nPKCs (for example PKCδ, Santos et al., 2007) and neurofibromin (von Kriegsheim et al., 2009) may actively mediate the long-lasting ERK activation that drives PC12 cell differentiation.

Neurofibromin is highly expressed in neurons with lower levels of expression detected in oligodendrocytes, Schwann cells, astrocytes, and other cell types (Li et al., 2001 and refs therein). Support for a specific role of neurofibromin in neuronal differentiation derives from several studies which show that its expression is developmentally regulated in the CNS and dorsal root ganglia in mouse and chick embryos, and that its peaks in expression coincide with the onset of neuronal differentiation (Vogel et al., 1995; Li et al., 2001). Studies in mice where neurofibromin has null expression after genetic ablation of central exons that render the protein unstable (functional knockout) have revealed an essential role for neurofibromin in the dependence of PNS neurons to neurotrophins (Vogel et al., 1995). Specifically, larger numbers of sensory and sympathetic neurons survive and continue to

differentiate in the absence of NGF in culture (Vogel et al., 1995; Vogel et al., 2000; Zhu et al., 2001), in a Ras-depended manner (Vogel et al., 2000). Interestingly, functional ablation of neurofibromin in CNS neurons via a synapsin-Cre approach does not result in altered morphology or survival rates (Zhu et al., 2001). Analysis in the PC12 cell model, utilizing siRNA-downregulation or overexpression of neurofibromin dominant-negative construct approaches, has yielded somewhat contradictory results. In one study, neurofibromin silencing did not affect NGF-induced differentiation, yet, it enabled EGF to partially promote differentiation (von Kriegsheim et al., 2009). In other studies, overexpression of a neurofibromin-based dominant negative construct, known to increase Ras-GTP and p-ERK levels, or siRNA-silencing of neurofibromin resulted in inhibition of NGF-induced differentiation (Ynoue et al., 2003; Patrakitkomjorn et al. 2008). These disparing results may stem from differences in the time period of siRNA silencing, extent of neurofibromin downregulation, and concentrations and times of NGF treatment. Nonetheless, these data attest to a developmental time-dependent function of neurofibromin during neuronal differentiation.

Many studies in neuronal cell lines and primary neurons have implicated most of PKC isoforms in survival and differentiation mechanisms (Mangoura et al., 1993; Hundle et al., 1997; Lallemend et al., 2005; Shirai et al., 2008). In their majority, PKCs have been shown to have a positive role in neuritic outgrowth; yet, in a recent large-scale analysis in hippocampal neurons PKCι and PKCη scored as potent negative neurite growth regulators (Buchser et al., 2010). PKCε on the other hand is regarded as the isoform involved in differentiation of both CNS and PNS neurons (Mangoura et al., 1993; Larsson, 2006; Mangoura, 1997; Hundle et al., 1997; Shirai et al., 2008). In fact, in the developing chick brain, it is the major isoform found in early post-mitotic, just starting to differentiate neurons (Mangoura et al., 1993). A significant number of studies on the pro-differentiation role of PKCε have been actually performed on NB cell lines, as will be presented in more detail in the following sections.

2.2 Neuroblastoma differentiating agents and underlying signalling mechanisms

2.2.1 Retinoids

Agents, neurotransmitters, growth factors, and neurotrophic factors used in culture models of NB differentiation are summarized in Table 1. Typical differentiating agents are the retinoids all trans-RA, 13-cis RA, and N-(4-hydroxyphenyl) retinamide (4-HPR, or fenretidine) (Reynolds & Lie, 2000), which cause growth arrest concomitantly with downregulation of MYCN expression, upregulation of an array of neuronal markers (neuron-specific enolase, neuropeptide Y, GAP43, MAP2, neurofilament-M, and synaptophysin), induction of neurite outgrowth, and increases in neurotransmitter biosynthetic enzyme activity and expression. The neurotransmitter phenotype induced by RA depends on the cell line, with cells developing sympathetic noradrenergic or cholinergic phenotypes (Edsjo et al., 2007 and refs. therein). Efforts to establish a consensus on what constitutes the RA-induced differentiation has recently led to recognition of a set of 10 genes as a potential signature and a more general yet reliable predictor of differentiation in NBs (Hahn et al., 2008). Moreover, a gene-expression high-throughput screening of small molecule libraries revealed a synergistic action of RA (all trans RA and 13-cis RA) and histone deacetylase inhibitors towards induction of NB differentiation, both in culture and in vivo (Hahn et al., 2008).

Agent	Phenotype/Markers	Signalling pathway	Cell lines/Comments/ References
Retinoids			
all-trans RA/13-cis RA	growth arrest, ↑ neuronal markers, morphology	RAR, PKCs, ATM, ERK (inconclusive)	Most NB cell lines, resistance, heterogeneity (see text)
4-HPR (fenretinide)	growth arrest apoptosis/necrosis variable differentiation	RAR-independent, ceramide, PKCs, ROS	RA-sensitive and resistant NB cell lines (1-3)
Phorbol esters			
TPA (12-O-tetradecanoyl-phorbol-13-acetate)	growth retardation ↑ neuronal markers morphology	PKCs (ERK, RARs)	best studied in SH-SY5Y (also SK-N-SH, SK-N-SN) (see text)
cAMP analogues			
dBcAMP	growth arrest ↑ neuronal markers morphology	PKA/Epac, CREB, PI3K/ERK	Most NB cell lines (see text)
Growth factors			
IGF-1/bFGF	↑ neuronal markers morphology	PKCs, ERK, RARβ	SH-SY5Y (4-5)
NGF	↑ neuronal markers morphology	TrkA, PKCs	TrkA/SH-SY5Y, IMR32 (5)
GDNF	growth inhibition cell cycle arrest ↑ neuronal markers	RET/TrkA	SH-SY5Y, LA-N-5 (6)
GPCR agonists			
Adenosine	↑ neuronal markers morphology	PKC, ERK, PKA (receptor-specific)	SH-SY5Y (7)
PACAP	↑ neuronal markers morphology	cAMP, ERK/p38	SH-SY5Y (8)
Other			
Uridine	growth retardation ↑ neuronal markers morphology	PKCε	LAN-5 (9)

Table 1. NB differentiating agents and signalling mechanisms studied; growth retardation refers to decreased proliferation rate; morphology refers to increased neuritic outgrowth measured as a percentage of neurite-bearing cells or average neurite length; neuronal markers refer to increases in neuron-specific enolase, NPY, GAP43, synaptophysin, and TH (mRNA or protein level); signalling pathway implication has been derived mostly by pharmacological and genetic manipulation studies. Only selected pathways are presented to best serve the focus of this chapter. Details of some of the experiments are discussed in the text and further information may be found in the respective references [1-3, (Edsjo et al., 2007; Janardhanan et al., 2009; Reynolds & Lie, 2000); 4-5, (Perez-Juste & Aranda, 1999; Fagerstrom et al., 1996); 6, (Peterson & Bogenmann, 2004); 7, (Canals et al., 2005); 8, (Monaghan et al., 2008); 9, (Silei et al., 2000)].

Studies with NB cell lines are subjected to some degree of heterogeneity in the response which may stem from: the passage number, culture conditions, concentration of agents, length of treatment, and certainly the read-out assay system. Thus conflicting reports are not rare, for example the NB cell line IMR32 has been described as resistant (Joshi et al., 2007),

sensitive (Holzel et al., 2010), or weakly responsive to RA (Guzhova et al., 2001). Similarly, SK-N-BE2 cells have been characterized as RA-resistant in a long-term 25-30 day colony formation assay (Holzel et al., 2010), and as sensitive when increased neuritic outgrowth was recorded after 2-4 days of exposure (Zeidman et al., 1999a, 2002). For RA, in particular, this is a significant issue, since prediction of resistance to retinoid therapy is very important in the clinical setting. Until recently, no predictive markers of RA responsiveness were established for clinical use. A connection with amplification of MYCN has been suggested (Reynolds et al., 2000), yet, MYCN-overexpressing stable SK-N-SH cells retain their capacity to differentiate in response to a variety of agents, including RA (Edsjo et al., 2004). Several other genes investigated in NBs have not come through as predictors, and even RARs have been questioned, because RA-resistant cells are sensitive to fenretinide (N-(4-hydroxyphenyl) retinamide), the synthetic retinoid that acts independently of RARs (Reynolds et al., 2000; Reynolds and Lie, 2000). More recently, neurofibromin deficiency in NB cells was proposed as a strong predictor of RA responsiveness, as shRNA silencing of neurofibromin was shown to confer resistance to retinoic acid independently of MYCN expression. Neurofibromin-deficient cells continue to proliferate in the presence of low concentrations of RA, bypassing check points for growth arrest and induction of differentiation, and exhibiting reduced expression of RA-target genes, Ret included (Holzel et al., 2010). Expression of Ret, the GDNF receptor, is very important for differentiation of NB cells (Peterson & Bogenmann, 2004; Esposito et al., 2008), especially through its action on upregulation of TrkA, a powerful and favorable prognostic marker in NB tumors (Edsjo et al., 2007; Brodeur, 2003).

The need for prognostic markers and therapeutic targets has strongly driven research on the signalling mechanisms that regulate RA-induced NB differentiation. Apparently RARs constitute a major requirement for the action of RA, as RA confers a significant upregulation of RARβ that drives gains in growth arrest and morphological NB differentiation (Maden, 2007). Besides the well-appreciated role of RARβ, other key signalling pathways appear to contribute to or modulate the RA-induced NB differentiation. These pathways are rapidly engaged upon addition of RA and often persist over a long time period. Thus, RA added to SH-SY5Y or SK-N-BE rapidly (within 20 min) activates the ATM kinase (Fernandes et al., 2007), which is a component of the DNA damage signal transduction pathway. ATM's autophosphorylation persists for over 4 days and correlates with enhanced ATM-dependent CREB phosphorylation and cell differentiation (Fernandes et al., 2007). Importantly, ATM inhibition or depletion does not prevent RA-induced upregulation of target genes, rather impairs cell survival (Fernandes et al., 2007).

A large body of evidence suggests a positive role of Ras, Raf, and prolonged ERK activation in neuronal differentiation in PC12 cells and several other cellular models (see section 2.1.2), but the situation is certainly more complicated when RA-induced NB differentiation is concerned. ERK is invariably activated by RA in the short- (Delaune et al., 2008) or long-term (Lee & Kim, 2004; Miloso et al., 2004), yet inhibition of the ERK activating kinase MEK by U0126 or PD98059 has yielded contradictory results on whether RA-induced ERK activation is necessary for neuritic outgrowth in SH-SY5Y and SK-N-BE(2)C cells (Miloso et al., 2004; Lee & Kim, 2004). Stable overexpression of RKIP (Raf kinase inhibitor protein), a scaffold protein crucial for Raf and ERK pathway activation, accelerated the rate of neuritic outgrowth and increased the expression of neuronal markers in response to RA in SH-SY5Y cells, all concurrent with sustained ERK activation (Hellmann et al., 2010). Furthermore, in

SH-SY5Y and LA-N-5 cells, ERK mediates an early (within 1h) RA-induced formation of promyelocytic leukemia nuclear bodies, an event associated with NB differentiation (Delaune et al., 2008). Apparently, ERK activity may impinge differentially on early and late gene regulation and inhibitor studies have not always addressed these issues with sufficient detail (Miloso et al., 2004; Lee & Kim, 2004; Delaune et al., 2008; Holzel et al., 2010). In addition, evidence from the various differentiation protocols and agents suggests that the transcription programs for neuritic outgrowth or expression of neuron-specific genes may be independently regulated in NB cells. In all, ERK activity appears to be crucial for the expression of neuron-specific genes, in the absence of a positive signalling stimulus for the induction of neuritic outgrowth (see below in section 2.2.2).

Besides its role as a differentiating agent, 13-cis-RA antagonizes significantly the cytotoxic effects of agents like etoposide and cisplatin (Hadjidaniel & Reynolds, 2010), which suggests another mechanism of impact of the RA responsiveness, this time involving the Ras/ERK pathway. For example, an inverse correlation between resistance to doxorubicin and ERK pathway activation has been suggested in SK-N-SH cells (Mattingly et al., 2001), while NFkB activation by doxorubicin and etoposide in SH-SY5Y cells, thought to be required for drug-induced toxicity, depends also on Ras and MEK activities (Armstrong et al., 2006). With the newly established role of neurofibromin in RA responsiveness and its direct implication in signalling through the Ras/ERK pathway for neuronal differentiation, neurofibromin may indeed contribute to some extent in (the prevention of) cytotoxic responses, an aspect not yet considered.

Experimental evidence from chemical inhibition or overexpression of dominant-negative mutants studies has implicated several PKC isoforms, mostly nPKCs, in RA-induced differentiation. RA-induced ERK activation in SH-SY5Y cells was significantly reduced in the presence of the c/nPKC inhibitor GF1092303X, as well as the RA-induced cell survival and neuritic outgrowth (Miloso et al., 2004). A peptide derived from the actin-binding site of PKCε has been shown to attenuate RA-induced neuritic outgrowth in SK-N-BE(2), while RA induced an increase of the cytoskeleton-associated PKCε pool (Zeidman et al., 2002). Besides PKCε, PKCθ and PKCδ have also been suggested to play a role in RA-induced differentiation (Nitti et al, 2010). In LAN-5 cells, RA-induced differentiation is inhibited by PKCθ-antisense oligonucleotides (Sparatore et al., 2000), while in SH-SY5Y cells, modulation of PKCδ activity by inhibition with rottlerin or overexpression of dominant-negative PKCδ suggested a positive role of PKCδ activity in SH-SY5Y differentiation via the function of NADPH oxidase system (Nitti et al, 2010).

2.2.2 Phorbol esters

Long-term treatment of SH-SY5Y cells with the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) has been extensively studied as a NB and human sympathetic neuron differentiation model, providing instrumental insights on the role of PKC in NB differentiation. TPA, acting as a DAG analog, directly binds and activates c/nPKCs, the main transducers of the TPA differentiation signal. Treatment of SH-SY5Y cells with low nanomolar concentrations of TPA results in the acute activation of PKCs and progressively drives cells towards a well-described sympathetic phenotype. This phenotype is characterized by increased neuritic outgrowth and upregulation of neuron-specific genes, such as GAP-43, neuropeptide Y, and tyrosine hydroxylase (Pahlman et al. 1981; Troller et al. 2001; Olsson & Nanberg 2001), increases in noradrenaline content (Pahlman et al., 1984;

Heikkila et al. 1993), and development of membrane excitability (Jalonen & Akerman, 1988). Besides SH-SY5Y and the parental cell lines SK-N-SH and SK-N-SN, IMR32 cells also respond to TPA treatment with induction of neuronal genes that specify neurotransmitter phenotypes (Mangoura et al., 2006b). PKC activation may mediate significant crosstalk with the RARs, as TPA induces the upregulation of RARβ expression in an nPKC/Ras/Raf-dependent manner (Perez-Juste & Aranda, 1999). Also in IMR32 cells, a synergistic transcriptional action of PKC and Ras with NF1 has been demonstrated in the induction of TH expression by TPA (Mangoura et al., 2006b).

The prominent role of PKCs in TPA-induced SH-SY5Y differentiation has been established in many studies and the most important aspects have been reviewed by Larsson, Pahlman, and co-workers (Larsson, 2006; Edsjo et al., 2007), where they note that PKCε is primarily responsible for TPA-induced differentiation, in particular for the induction of neuritic outgrowth. Characteristically, transfections with PKC constructs have suggested a dominant role of the PKCε regulatory domain as well as of its, unique amongst other PKCs, actin-binding properties in inducing neuritic outgrowth (Zeidman et al., 1999a, Zeidman et al., 2002) [2]. Thus, the PKCε pro-differentiating effects may involve mechanisms that do not directly implicate its kinase activity (Zeidman et al., 1999a). The kinase activity is also very important, as c/nPKC inhibitors, such as GF1092303X and Ro-318220, have been repeatedly shown to effectively inhibit TPA-induced ERK activation (Olsson et al., 2000; Troller et al., 2001; Leondaritis et al., 2009), neurofibromin phosphorylation (Leondaritis et al., 2009), and, TPA-induced neuritic outgrowth, expression of neuronal markers, and increase of neurotransmitter content (Heikkila et al., 1993; Fagerstrom et al., 1996; Troller et al., 2001; Olsson et al., 2000). Yet, further elucidation of the roles of individual PKCs would greatly benefit from more specific ATP-targeted inhibitors of cPKCs and nPKCs (Way et al., 2000). In this context, studies that used long-term inhibition of cPKCs with Go6976 to assess proliferation and survival of NB cells need to be re-evaluated, in lieu of recent studies suggesting that (a) Go6976 is a highly potent inhibitor of Aurora A and B kinases (Stolz et al., 2009), (b) Aurora A is overexpressed in most NB cell lines and stage 3-4 NB tumors and (c) Aurora A inhibitors have broad anti-NB activity (Maris, 2010 and refs therein).

An important feature of TPA-induced differentiation of SH-SY5Y cells is the apparent bifurcation of proximal signalling requirements for neuritic outgrowth or neuronal marker expression. Indeed, inhibition of ERK activation by PD98059 abolishes TPA-induced upregulation of neuropeptide Y and GAP-43, but has no effect on neuritic outgrowth (Olsson & Nanberg, 2001). In agreement, inhibition of PKCβI by LY379196 reduces TPA-induced ERK activation, and expression of GAP43 and neuropeptide Y, yet has no effect on neuritic outgrowth (Troller et al., 2001). Collectively, most studies converge on two important aspects: (i) the occurrence of an active cPKCα and cPKCβI-ERK axis in regulation of the early (within 24h) induction of neuronal markers and (ii) the mandatory role of PKCε, via both catalytic and scaffolding mechanisms, in the later induction of neuritic outgrowth and maintenance of differentiation.

2.2.3 Cyclic AMP analogues

cAMP analogues, such as bi-butyryl-cAMP (dbcAMP), have been long used, often in combination with bromodeoxyuridine (BrdU), to induce NB differentiation. Many neuronal

[2]Florakis et al., 2006, Federation of European Neuroscience Societies (FENS) Forum 3:A051.5

cell lines, like PC12 and NB cells (SH-SY5Y and IMR32), respond to these powerful intracellular signal transducers with increased neuritic outgrowth and upregulation of neuronal markers, such as GAP43 and TH (e.g Christensen et al., 2003; Birkeland et al., 2009). The effect of cAMP analogues may also be reproduced with forskolin, a direct activator of adenylyl cyclase (AC). Forskolin models the established ability of many GPCR agonists coupled to Gs/AC activation to drive differentiation via increased production of cAMP in various NB and other cellular contexts (Monaghan et al., 2008; Canals et al., 2005). Two major pathways serve as proximal transducers of the cAMP signal: the Epac1/2 (exchange protein activated by cAMP)/Rap GTPase and the PKA pathways (Bos, 2006), both of which may contribute to differentiation (Christensen et al., 2003; Birkeland et al., 2009). In addition to PKA and Epac, PI3K has also been implicated in cAMP-induced differentiation of NB cells as well as in the maintenance of elongated neurites (Sanchez et al., 2004).

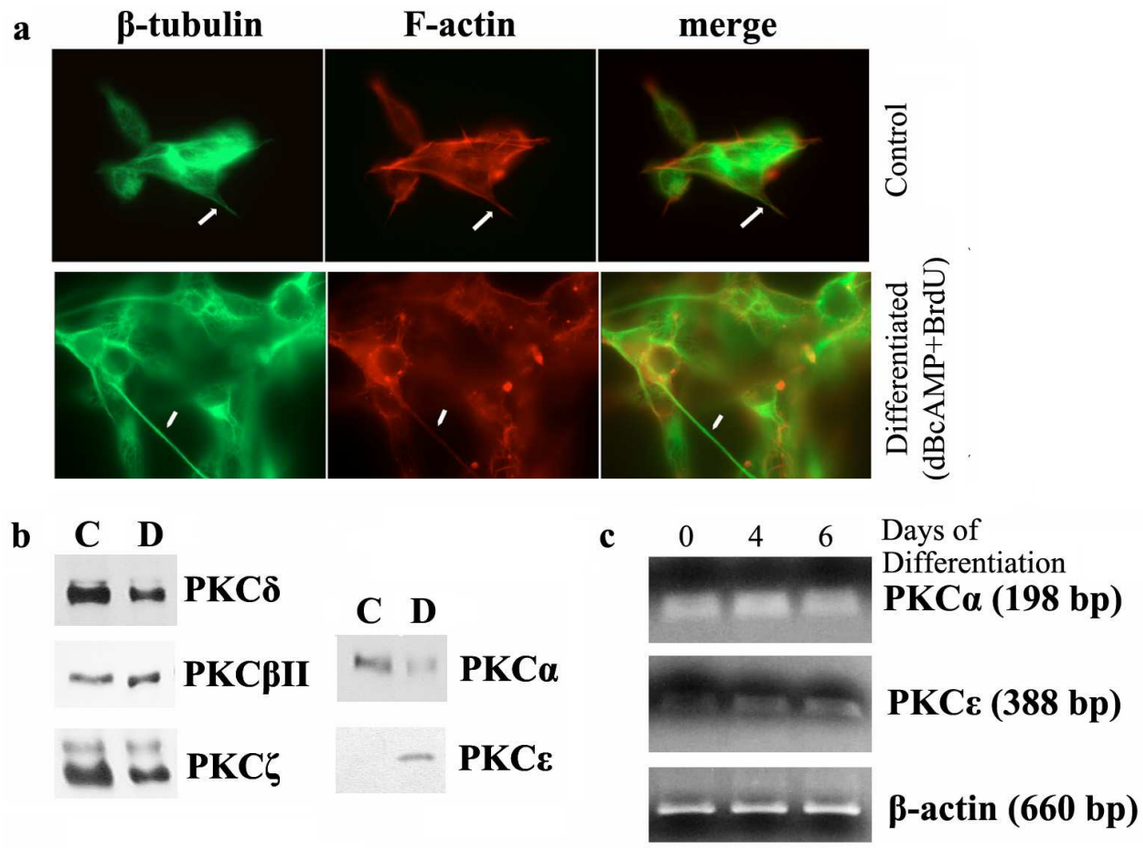


Fig. 1. Upregulation of PKCε and downregulation of PKCα during cAMP-induced differentiation of IMR32 cells. Cells were incubated in MEM plus 10% heat-inactivated FBS in the absence (control, C) or presence (D) of dBcAMP+ BrdU. Fixation, antibodies, immunostaining, and microscopy (a), as well as Western blotting (b) and RT-PCR (c) were performed as described (e. g., Mangoura et al., 1993; Li et al., 2001; Mangoua et al., 2006b; Leondaritis et al., 2009)

A typical example of cAMP-differentiated IMR32 cells is illustrated in Fig. 1a. After 4-6 days of 1 mM dBcAMP and 2.5 μM BrdU cells have acquired pyramidal-shaped cell bodies and

elongated neurites that often extend up to 4-5 times the cell body length. Double staining for F-actin and βIII-tubulin reveals the extended organization of microtubules into organized, axon-like bundles, quite rich in F-actin (Fig. 1a, arrows in upper versus arrows in lower panels). PKCs have not been widely implicated in cAMP-induced NB differentiation, yet our analysis of the protein levels of PKC isoforms reveals great differences in control and differentiated cells (Fig. 1b). Specifically, the levels of the cPKC βII, and nPKC δ or ζ remain unaltered, cPKCα is extensively downregulated and nPKCε upregulated (Fig. 1b). Downregulation of PKCα is probably a post-translational event, as its mRNA levels remain constant throughout dBcAMP+BrdU exposure (Fig. 1c, 0 versus 4 and 6 days). In contrast, PKCε mRNA expression is significantly and progressively upregulated over the same time period (Fig. 1c). From these observations it is clear that cPKCα and nPKCε may impinge on cAMP-differentiation pathways, in a similar manner to that described for RA and TPA-differentiation pathways in sections 2.2.1 and 2.2.2.

2.3 Aspects of PKC regulation in neuroblastoma differentiation

2.3.1 cPKCs vs nPKCs and differentiation

Ample evidence exists for PKC requirement in NB differentiation by TPA and RA (Table 1, sections 2.2.1 and 2.2.2). Furthermore, PKC activity is implicated in NB differentiation mechanisms set in motion by growth factors, neurotrophic factors, and GPCR agonists (Fagerstrom et al., 1996; Silei et al., 2000; Canals et al., 2005; Monaghan et al., 2008) (Table 1). In some instances, PKCε has been identified as the crucial isoform (e.g. Silei et al., 2000; Fagerstrom et al., 1996), but there is still some controversy for the contribution of other nPKCs, namely PKC δ and θ. PKCθ, expressed occasionally in NB cells or tumors (Zeidman et al., 1999b), may contribute to apoptotic pathways in SK-N-BE(2) and SH-SY5Y cells (Schultz & Larsson, 2004; Schultz et al., 2003). Unlike θ, δ is commonly expressed (Zeidman et al., 1999b), yet again, transfection studies with full-length, catalytically inactive, or active PKCδ variants have revealed a pro-apoptotic role (Schultz et al., 2003; Schultz & Larsson, 2004). A major role of PKCδ in the sensitization of NB cells (SH-SY5Y and SK-N-BE(2C)) to etoposide (Marengo et al., 2011) was just recently described, validating similar observations in other cell types (Griner and Katanietz, 2007). The function of aPKCs in NB differentiation is not clear at present, except recent indications that inhibition of PKCι has pro-apoptotic, and antiproliferative effects (Pilai et al., 2011).

The balance between abundance and activity of cPKCs versus nPKCs during initiation (early responses) or maintenance of NB differentiation has emerged as an important question. cPKCs are thought to be involved in early responses, such as induction of neuronal markers and commitment of NB cells to survival pathways. nPKCs appear to control the long term phenotypic result consisting of elaboration of dentrites, as well as stabilization of the transcriptional networks that specify differentiation and neurotransmitter phenotype acquisition. This distinct mode of action may be best appreciated by the altered regulation of expression levels of the cPKC α and the nPKC ε during cAMP-induced differentiation of IMR32 cells (Fig. 1). This shift in balance of PKCα/PKCε expression during differentiation has been repeatedly observed in developmental studies and the analysis of the periods of neurogenesis and neurodifferentiation, or in culture models that recapitulate these periods (Mangoura et al., 1993; Battaini et al., 1994). Furthermore, it has been suggested that growth factor-induced differentiation of SH-SY5Y may occur even when PKCα is ablated after treatment with high (1 μM) TPA concentrations (Fagerstrom et al., 1996). Remarkably,

experiments in IMR32 cells directly corroborate and expand this notion. As shown in Fig. 2, direct, specific downregulation of PKCα expression with phosphorothioate-modified antisense oligonucleotides (5'-GGGACCATGGCTGACGT-3', 15 μg/mL x 5 days) results in a differentiated phenotype, indistinguishable from that caused by dBcAMP+BrdU (Fig. 1). Thus, suppression of PKCα alone may suffice for the induction of morphological differentiation in NB cell lines.

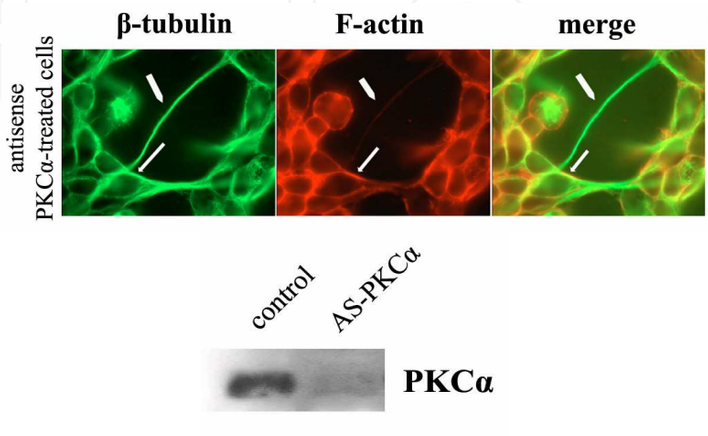


Fig. 2. Specific downregulation of PKCα induces differentiation in IMR32 cells. PKCα translation was silenced with antisense-PKCα oligonucleotides. Arrows indicate different long, tubulin-rich processes and intense cortical actin staining (upper panel). Downregulation of PKCα protein levels was confirmed by Western blotting (lower panel). Methods were performed as in Fig. 1

2.3.2 PKCs and cytoskeletal changes during NB differentiation

The input of PKC activation on cytoskeletal changes associated with neuritic outgrowth during NB differentiation in all three cytoskeleton systems, namely microtubules, F-actin, and intermediate filaments is well established. The F-actin cortical cytoskeleton may be viewed, in a general sense, as a direct target of the differentiation process (Mangoura et al., 1997). Among prominent PKC substrates of particular relevance are proteins that directly bind to F-actin, such as MARCKS, GAP43, adducin, fascin, and ERM proteins (Larsson, 2006). MARCKS and GAP43 have a pivotal role in remodeling the F-actin cytoskeleton during neurite outgrowth, growth cone motility, and synapse formation (Laux et al., 2000; Larsson, 2006). Neurofibromin is another PKC substrate that may associate with the F-actin cytoskeleton in neurons and SH-SY5Y cells (Li et al., 2001; Mangoura et al., 2006a).

Analysis of cytoskeletal protein localization after a prolonged (24h) treatment of IMR32 cells with TPA reveals also a sustained recruitment of neurofibromin in Triton X-100 insoluble fractions (HP lanes, Fig. 3) where cortical cytoskeleton proteins reside; concurrently, neurofibromin is not detected in the urea-soluble fractions (UF lanes, Fig. 3), where cytosolic organelle and nuclear proteins are found. At the same time, there is substantial loss of the intermediate filament protein vimentin in Triton X-100-soluble (cytosol) and -insoluble fractions (T2 and HP lanes, respectively), consistent with its downregulation upon differentiation of NB cells (Yabe et al., 2003). In parallel, vinculin, an F-actin binding protein and developmentally-regulated (Cheng et al., 2000) PKC substrate (Mangoura, 1997),

becomes enriched in cytoplasmic actin fractions (T2 lanes), suggestive of the extended reorganization of the actin cytoskeleton at the onset of neuritic outgrowth.
PKCs may serve also as upstream regulators of other signalling modules that modify cortical actin cytoskeleton proteins. For example, nPKCs, and specifically PKCε, are necessary for Src/FAK-dependent formation of active Cas/Crk complexes in differentiated SH-SY5Y cells (Fagerstrom et al., 1998; Bruce-Staskal & Bouton, 2001) and in neurons (Mangoura, 1997). Importantly, Src activation and phosphorylation is a PKCε-sensitive, long lasting (>16h) event during TPA- and IGF1/bFGF-induced differentiation of SH-SY5Y cells, suggesting the engagement of this pathway in the extensive remodeling of actin cytoskeleton during elongation and elaboration of neurites (Fagerstrom et al., 1998). Notably, PKCε was recently shown to organize a proximal signalling protein complex containing Src and Fyn tyrosine kinases, essential for downstream ERK activation in neurons (Asimaki & Mangoura, 2011), and in SH-SY5Y cells (Asimaki et al., 2007).

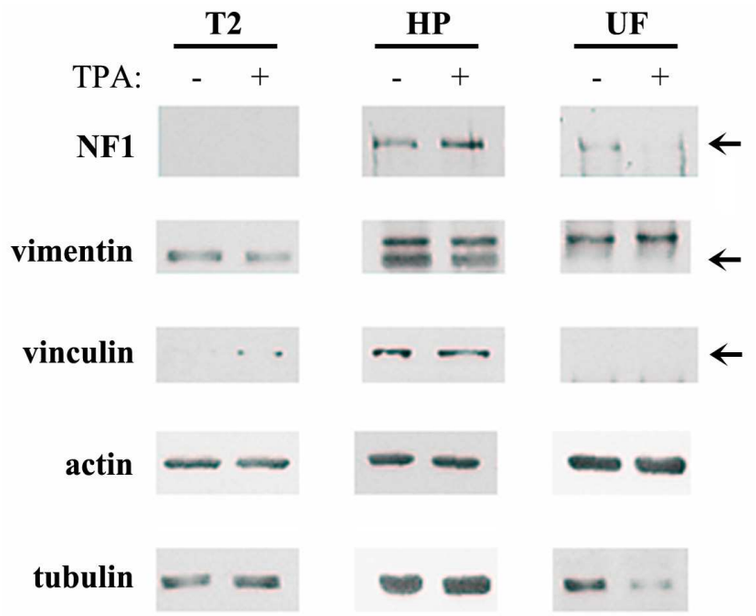


Fig. 3. Changes in cytoskeletal protein localization during early phases of TPA-induced differentiation in IMR32 cells. Cells were treated with 100 nM TPA for 24 h and cell lysates were fractionated into Triton X 100-soluble cytoplasmic actin fractions (T2), Triton X 100-insoluble cortical actin fractions (HP), and urea-soluble fractions (UF, representing also nuclear proteins), as described (Fox et al., 1993). Protein abundance in the fractions was analysed using Western blotting and the indicated antibodies (Mangoura, 1997; Li et al., 2000; Mangoura et al., 2006a).

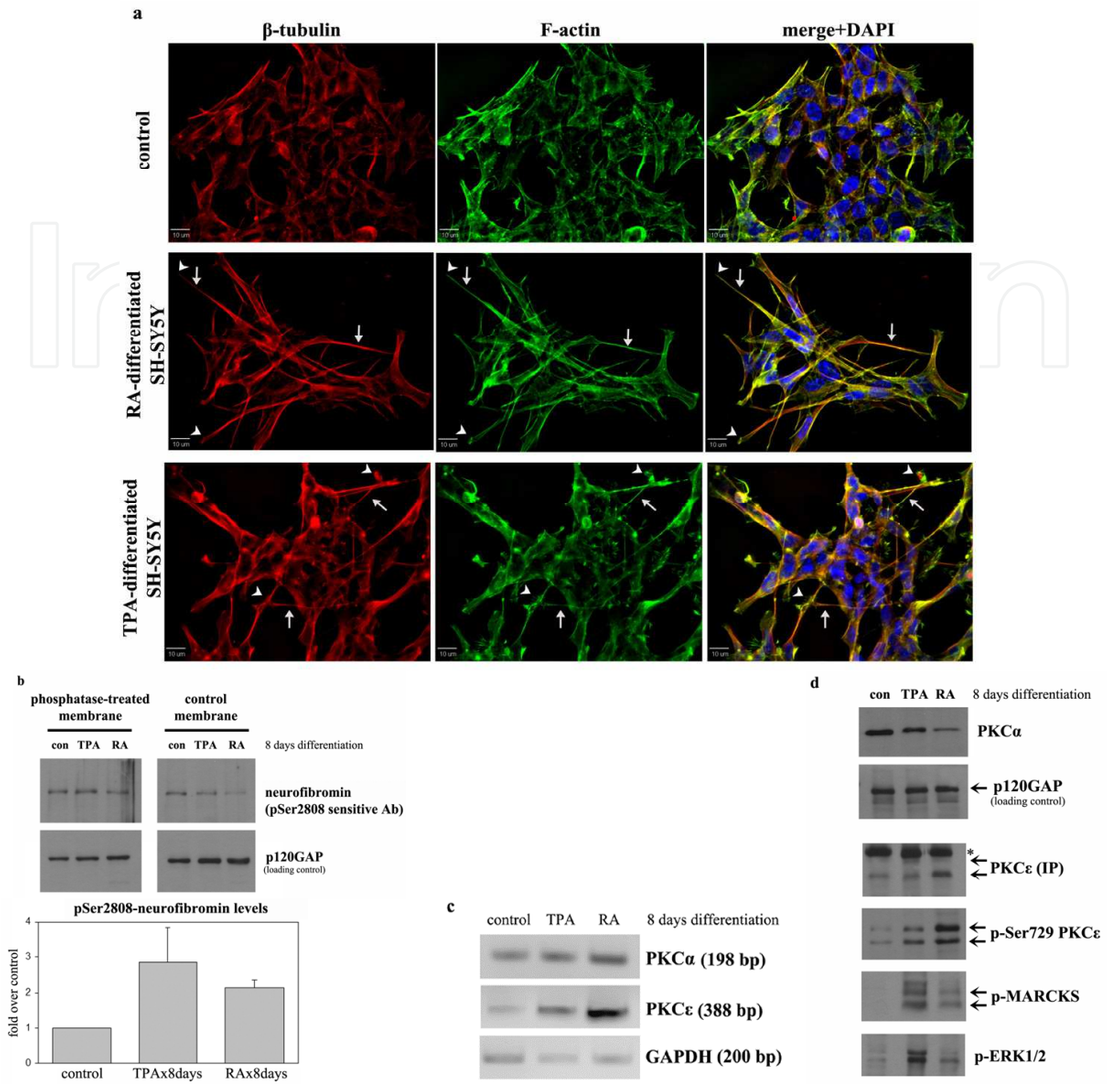
Independent studies on gene expression profiles in advanced NB tumors have shown that many genes involved in Fyn signalling and organization of the F-actin cytoskeleton are downregulated (Berwanger et al., 2002). The importance of Fyn, in particular, was further validated when expression of active Fyn elicited differentiation and growth arrest in NB cells (Berwanger et al., 2002). These studies collectively highlight the tight inter-regulation between the abundance and activation of PKCε and Src/Fyn, and the re-organization of the

F-actin cytoskeleton during NB differentiation. Further support for this aspect is lend by the high concentration of PKCε in growth cones of differentiated SH-SY5Y and SK-N-BE(2) cells (Fagerstrom et al., 1996; Zeidman et al., 1999a, 2002).

2.3.3 Differentiating agents regulate a functional PKCα/PKCε balance that controls neurofibromin phosphorylation and downstream ERK signalling in neuroblastoma cells

Neurofibromin is currently emerging as an important signalling protein positioned at the intersection of NB differentiation pathways, as A, it acts as a rheostat for the activation of the Ras/ERK pathway (Cichowski et al., 2003; Mangoura et al., 2006a, b), B, it is a NB tumor suppressor with only 50% of NB cell lines bearing two normal alleles, and 6% of primary NBs bearing genomic NF1 aberrations (Holzel et al., 2010), C, it is a PKC substrate (Mangoura et al., 2006a) and it is extensively and persistently phosphorylated at a PKC-specific site, the C-terminal Ser2808, in TPA-differentiated SH-SY5Y cells (Leondaritis et al., 2009), D, it provides responsiveness to RA (Holzel et al., 2010). Neurofibromin has been also implicated in cAMP signalling in other cell types (Rubin & Gutman, 2005) and this suggests that it may impinge on cAMP-induced NB differentiation as well. Yet, the effects of NB differentiating agents on neurofibromin abundance (Cichowski et al., 2003), phosphorylation (Izawa et al., 1996; Mangoura et al., 2006a; Leondaritis et al., 2009), and RasGAP activity (Mangoura et al., 2006a), all critical aspects of its function as a Ras/ERK pathway modulator, have not been thoroughly addressed with the exception of TPA-differentiated SH-SY5Y (Leondaritis et al., 2009). Long-term treatment of SH-SY5Y with TPA or RA results in differentiation with variations in the acquired neurotransmitter phenotypes (Pahlman et al., 1984), yet morphologically, all cells long extensions, heavily decorated with β-tubulin (Fig. 4a, arrows) and F-actin in their growing neuritic tips (Fig. 4a, arrowheads), these features are virtually absent in control cells (Fig. 4a, upper panel).

It is a striking event that neurofibromin is significantly phosphorylated (2-3 fold over control) at the PKC-specific, C-terminal Ser2808 site in SH-SY5Y cells even after 8 day of differentiation with TPA or RA (Fig. 4b). Thus, persistent PKC-specific neurofibromin phosphorylation appears to be a common feature of both TPA- and RA-differentiated SH-SY5Y cells. Neurofibromin may be acutely phosphorylated after stimulation by EGF or TPA by either PKCα or PKCε in neurons (Mangoura et al., 2006a). In SH-SY5Y cells, TPA-dependent, acute neurofibromin Ser2808-phosphorylation appears to be mediated primarily by cPKCs (Leondaritis et al., 2009). In the long term, PKCα mRNA levels are modestly increased in TPA- and RA-differentiated cells (2-3 fold over control), concurrently with extensive upregulation of PKCε mRNA levels (>10-fold over control) (Fig. 4c). At the protein level, however, differentiation is accompanied by reciprocal changes in PKCα and PKCε abundance and activity: PKCα protein levels are substantially downregulated, while PKCε protein levels are significantly upregulated, at least in RA-differentiated cells (Fig. 4d). Most importantly, phosphorylation of PKCε at Ser729, the hydrophobic motif residue, which is essential for priming the catalytic competence of PKCε (Cameron et al., 2009; Freeley et al., 2011), is significantly increased in both TPA- and RA-differentiated cells (Fig. 4d). Furthermore, phosphorylation of MARCKS, which can reliably serve as a proxy marker for total PKC activity, is also significantly increased in TPA- and RA-differentiated cells (Fig. 4d).



a. Cells were incubated in RPMI+10% HI-FBS in the absence (control, upper panel) or presence of 10 μM RA (middle panel), or 20 nM TPA (lower panel) for 8 days. Fixation and staining was performed as in Fig. 1. Images are projections of 5 0.5μm Z-stacks, deconvoluted with the nearest-neighbor algorithm (Slidebook software, Neurosciences Imaging Facility). b. Ser2808 phosphorylation of neurofibromin was studied with the use of sc-67 antibody in Western blot-phosphatase assays as described (Leondaritis et al., 2009). Note the significant gains in immunoreactivity to sc-67 in TPA- and RA-treated samples on membranes treated with phosphatase (left panel) that define the level of phosphorylation of neurofibromin on Ser2808. Quantification suggests a 2-3 fold increase of neurofibromin's Ser2808 phosphorylation in differentiated cells (lower panel). c) mRNA levels of PKCα, PKCε, and GAPDH were measured as described previously (Mangoura et al., 2006b). d) Abundance and phosphorylated forms of indicated proteins were assayed by Western blotting in total cell lysates; IP indicates that PKCε was immunoprecipitated from cell lysates and asterisk a crossreactive protein in the immunoprecipitates. Antibodies for p120GAP, p-Ser729 PKCε, p-MARCKS and p-ERK1/2 were as in Leondartis et al., 2009 and Asimaki & Mangoura, 2011.

Fig. 4. TPA and RA-differentiated SH-SY5Y cells exhibit similar patterns of morphology, neurofibromin phosphorylation, PKCα/PKCε expression and activation, and PKC downstream signalling.

Thus, differentiation in SH-SY5Y cells is characterized by intense, PKCε-dependent signalling; this may impact directly on phosphorylation and activation of downstream targets, such as neurofibromin (Fig. 4b) and ERK (Fig. 4d). In aggregate, this experimental evidence suggests that PKCε activation, neurofibromin Ser2808-phosphorylation, and ERK activation are all long-term effects, clearly associated with NB differentiation. Amongst these signal transduction, transcriptional, and translational events, regulation of PKCε arises as a nodal and fundamental feature of the differentiation process itself.

3. Conclusions

Regulation of long-term signalling that confers long lasting gene expression, and elicits and maintains differentiation in NB cells has yet to be fully explored. Moreover, the acute versus prolonged changes in posttranslational modifications of proteins within a signalling pathway that would support the fine tuning of pathway's output towards cell differentiation are still largely unresolved. NB cells constitute a unique model to address these issues, in hope that understanding the differentiation mechanism may provide predictors for NB therapy and clues for novel druggable targets or for increased efficacy of existing drugs. In this chapter we focused on PKCs (PKCε), neurofibromin, and the ERK pathway as important components of the action of differentiating agents in NB cells. Our experimental observations may well apply to most NB cells as the two cell lines used, IMR32 and SH-SY5Y, do not share a common pattern of genetic alterations: IMR32 cells are MYCN-positive and have wild-type ALK, while SH-SY5Y are MYCN-negative and have an activating mutation in ALK. Another focal point discussed for its important clinical implications, is the involvement of the tumor suppressor neurofibromin in RA- and TPA-induced differentiation.

PKCε is expected to impact significantly on NB differentiation through both its catalytic and scaffolding properties which may operate simultaneously. Apparently, cPKCs and nPKCs may be important for NB cell proliferation and apoptosis, and these may be regulated by PKCε. Also of clinical importance is the possibility that nPKCs (PKCδ, ε or others) may modulate NB cell responses to cytotoxic drugs. A second point concerning PKCε is its characterization as an oncogene in non-neural tumors (Basu & Sivaprasad, 2008). This PKCε eccentricity is shared with other members of the signaling mechanisms involved in NB differentiation, for example with H-Ras, a well-known oncogene when mutated, yet a favorable prognostic marker in MYCN-negative NB tumors when overexpressed, or TrkA, an oncogene and yet a favorable predictor when highly expressed in NBs (Brodeur, 2003). Both latter proteins are crucial for neuronal differentiation and survival in different cellular models, as PKCε. Therefore, we post the hypothesis that increases in PKCε in non NB tumors may reflect the cell's response to increase its differentiation.

In summary, novel approaches to treat NB in future are likely to be based on the continuing elucidation of the underlying signalling pathways that govern NB cell proliferation, apoptosis and differentiation, including, but not limited to, the PKC/ neurofibromin/ Ras/ ERK pathway. Combinatorial and multimodal therapies towards maximum efficacy and low toxicity will critically depend on the integration and implementation of this knowledge in further preclinical and clinical studies.

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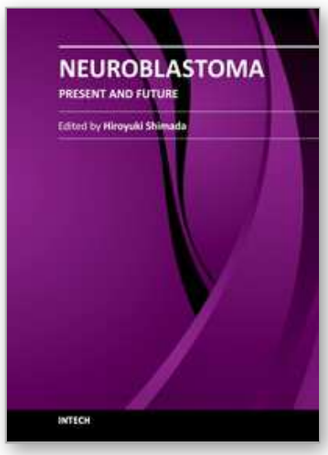

Neuroblastoma - Present and Future

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Neuroblastoma, once called "enigmatic", due to "unpredictable" clinical behaviors, is composed of biologically diverse tumors. Molecular/genomic properties unique to the individual tumors closely link to the clinical outcomes of patients. Establishing risk stratification models after analyzing biologic characteristics of each case has made a great success in patient management. However, the trend of improving survival rates in neuroblastoma over the last 30 years has started to level off, and currently available treatment modalities have almost reached to their maximized intensity. Furthermore, aggressive treatment causes significant long-term morbidities to the survivors. We really need to make the next step to the level of personalized medicine with more precise understanding of neuroblastoma biology. This book includes useful data and insights from the world's experts in this field. I believe this book can make an excellent contribution to all the investigators working hard and fighting for the children stricken by this disease.

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