Activation of Both Protein Kinase A (PKA) Type I and PKA Type II Isozymes Is Required for Retinoid-Induced Maturation of Acute Promyelocytic Leukemia Cells

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ABSTRACT

Acute promyelocytic leukemia (APL) is characterized by granulopoietic differentiation arrest at the promyelocytic stage. In most cases, this defect can be overcome by treatment with all-trans-retinoic acid (ATRA), leading to complete clinical remission. Cyclic AMP signaling has a key role in retinoid treatment efficacy: it enhances ATRA-induced maturation in ATRA-sensitive APL cells (including NB4 cells) and restores it in some ATRA-resistant cells (including NB4-LR1 cells). We show that the two cell types express identical levels of the Caα catalytic subunit and comparable global cAMP-dependent protein kinase A (PKA) enzyme activity. However, the maturation-resistant NB4-LR1 cells have a PKA isozyme switch: compared with the NB4 cells, they have decreased content of the juxtanuclearly located PKA regulatory subunit Ια and PKA regulatory subunit Ιβ, and a compensatory increase of the generally cytoplasmically distributed PKA-RIα. Furthermore, the PKA regulatory subunit ΙΙ exists mainly in the less cAMP-responsive nonautophosphorylated state in the NB4-LR1 cells. By the use of isozyme-specific cAMP analog pairs, we show that both PKA-I and PKA-II must be activated to achieve maturation in NB4-LR1 as well as NB4 cells. Therefore, special attention should be paid to activating not only PKA-I but also PKA-II in attempts to enhance ATRA-induced APL maturation in a clinical setting.

Introduction

Acute promyelocytic leukemia (APL) is characterized by an arrest of neutrophile granulocyte maturation at the promyelocytic stage and the reciprocal chromosomal translocation t (15,17), which fuses the promyelocytic leukemia (PML) and the retinoic acid receptor-α (RARα) (Rowley et al., 1977), resulting in the formation of a chimeric gene encoding the PML-RARα fusion protein (Borrow et al., 1990; de The et al., 1991). The unique sensitivity of APL cells to granulocyte differentiation induced by all-trans retinoic acid (ATRA) has been successfully exploited for the treatment of this leukemia. Despite the great success of ATRA combined with chemotherapy in APL clinical management, a significant number (5–30%) of patients develop resistance to ATRA and relapse (Ferrara, 2010). ATRA-induced APL differentiation can be enhanced by activators of cAMP signaling, and some primarily ATRA-resistant APL cells can be induced to differentiate if ATRA is combined with cAMP analogs (Ruchaud et al., 1994; Guillemin et al., 2002; Kamashev et al., 2004).

The major effector of cAMP is the cAMP-dependent protein kinase A (PKA). At low cAMP concentration, PKA exists as an inactive tetrameric holoenzyme composed of two regulatory (R) and two catalytic (C) subunits. Activation of PKA occurs when four molecules of cAMP bind to the R subunits, promoting the dissociation of the PKA holoenzyme into two active catalytic subunits and a dimer of regulatory subunits (Tasken et al., 1997). There are two types of PKA, which differ in their regulatory subunits (R subunits): Ια or Ιβ for PKA type I (PKA-I) and Ια or Ιβ for PKA type II (PKA-II) (McKnight

ABBREVIATIONS: APL, acute promyelocytic leukemia; ATRA, all-trans retinoic acid; BSA, bovine serum albumin; 2-Cl-8-AHA-cAMP, 2-chloro-8-aminoheptylamino-cAMP; Ν9-Bz-8-Pip-cAMP, Ν9-benzoyl-8-piperidino-cAMP; 2-Cl-8-MA-cAMP, 2-chloro-8-methylamino-cAMP; ΜΗ-DCFDA, 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester; 8-CPT-cAMP, 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphorothioate, DAPI, 4,6-diamidino-2-phenylindole; DCF, dihydrodichlorofluorescein diacetate; Epac, exchange protein activated by cAMP; Gaf, 4,6-diaminidine-2-phenylindole; GSH, glutathione; H2O2, hydrogen peroxide; PKI, protein kinase A inhibitor fragment 6-22 amide; PMA, phorbol 12-myristate 13-acetate; PML, promyelocytic leukemia; RARα, retinoic acid receptor-α; Sp, 5,6-di-CI-cBIMPS, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3',5'-cyclic monophosphorothioate, Sp-isomer.
et al., 1988; Skalhegg and Tasken, 1997). The PKA regulatory subunits define the sensitivity to cAMP (Robinson-Steiner et al., 1984; Rannels et al., 1985; Woodford et al., 1989; Zhang et al., 2012) and localize PKA at various subcellular sites through interaction with A-kinase anchoring proteins (Skroblin et al., 2010). Furthermore, only PKA-II but not PKA-I undergoes autophosphorylation (Rosen and Erlichman, 1975), which serves as a “feed-forward” signal by enhancing the cAMP responsiveness of PKA-II (Kopperud et al., 2003; Martin et al., 2007).

The NB4 cell line, a model of APL (Lanotte et al., 1991), and its variant sublines, NB4-LR1 and NB4-LR2, which are resistant to ATRA-induced maturation, offer an in vitro model to study ATRA-induced differentiation signaling pathways in APL cells and the molecular mechanisms of ATRA resistance (Duprez et al., 2000; Guillemin et al., 2002). The PKA activity appears to tune the ATRA-sensitivity of NB4 wild-type cells. NB4 cells, like APL cells in patients, do not differentiate at physiologic (1.0–10 nM) concentrations of ATRA but do undergo differentiation in response to pharmacologic doses (0.1–1.0 μM) of ATRA. The NB4 cells lose ATRA responsiveness when the basal PKA activity is inhibited by either R subunit–directed competitive antagonists of PKA dissociation (Ruchaud et al., 1994), or by active site–directed inhibitors of the C subunits (Zhao et al., 2004). Conversely, sustained increase of the endogenous level of cAMP renders NB4 cells responsive to physiologic levels of retinoids (3–10 nM) (Quenech’Du et al., 1998). In ATRA-induced maturation-resistant NB4-LR1 cells, maturation has been achieved by sequential or simultaneous treatment with ATRA and cAMP analogs (Ruchaud et al., 1994). The in vitro studies on NB4 cell lines have been confirmed in vivo using PML-RAR transgenic mouse models and so far one APL patient (Guillemin et al., 2002). Recently, it has been proposed that cAMP/PKA, by inducing Ser873 phosphorylation of the PML-RARA fusion protein, could contribute to eradicating leukemia-initiating cells in vivo (Nasr et al., 2008). These reports indicate a significant role of the cAMP-PKA pathway not only in the differentiation of granulocytic cells but also in the eradication of leukemia-initiating cells in vivo. Therefore, understanding the mechanism of action of cAMP/PKA signaling in APL cells is of interest not only for the treatment of APL but also for the understanding of its etiology and progression.

The NB4-LR1 cell, which requires exogenous stimulation of cAMP signaling in addition to ATRA treatment for maturation, provides a valuable tool for deciphering the complex PKA/retinoid crosstalk required for APL cell maturation. A previous study suggested that the differential response of NB4 and NB4-LR1 cells to ATRA treatment was explained by a lower level of basal level of cAMP in NB4-LR1 than in NB4 wild-type cells (Zhao et al., 2004). Here, we show that NB4-LR1 cells also differ from NB4 cells by having downregulated PKA-IIα and PKA-IIβ, resulting in a PKA-II to PKA-I isozyme switch. Moreover, the sustained activation of both types of PKA by cAMP analogs is required to allow ATRA-induced maturation of APL cells.

**Materials and Methods**

**Reagents.** The all-trans retinoic acid (ATRA), 8,4-(4-chlorophenylthio) adenosine 3′,5′-cyclic adenosine monophosphate (8-CPT-cAMP), PKA inhibitory peptide (protein kinase A inhibitor fragment 6-22 amide, PKI), p-nitro blue tetrazolium chloride (NBt), and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (St. Louis, MO). The 2-chloro-8-methylamino-cAMP (2-CI-8-MA-cAMP), 8-piperidino-cAMP (8-Pip-cAMP), N′-mono-tet-butylcarbamoyl-cAMP (N′-MBC-cAMP), 5,6-dichloro-1-β-D-ribofuranylimidazole-3′,5′- cyclic monophosphorothioate, S-iso-isomer (Sp-5,6-DCl-BIPS), and the Epac (exchange protein activated by cAMP-specific) cAMP analog 8-(p-meO-P)t-O2′-Me-cAMP, #M0134 were obtained from Biolog (Bremen, Germany). The new cAMP analogs 2-chloro-8-Aminohexylamino-cAMP (2-CI-8-AHA-cAMP) and N′-benzoyl-8-piperidino-cAMP (N′-Bz-8-Pip-cAMP) were synthesized as described elsewhere (Huseby et al., 2011).

**Cell Lines and Cell Culture.** The NB4, NB4-LR1, and NB4-LR2 cell lines were cultivated as previously reported elsewhere (Lanotte et al., 1991) in RPMI 1640 medium supplemented with 10% fetal bovine serum (PAA Laboratories, Pasching, Austria), penicillin (100 U/ml), streptomycin (100 μg/ml), and sodium bicarbonate (0.75 mg/ml), and were incubated at 37°C in a 5% CO2 atmosphere.

**In Vitro PKA Assay.** PKA activity in NB4 cells lysates was measured using a PepTag nonradioactive protein kinase assay kit (Promega, Madison, WI). This assay is based on the in vitro phosphorylation of a fluorescent PKA-specific peptide substrate (kemptide). Briefly, NB4 cells were washed twice with ice-cold phosphate-buffered saline (PBS), suspended in 10 mM potassium phosphate (pH 7.2), 0.3 mM EGTA, 0.1% Triton-X-100, 2.5 μM NaF, 0.2 mM NaN3, 0.5 mg/ml trypsin inhibitor (lysis buffer) supplemented with 1% protease inhibitor cocktail (P8340) (Sigma-Aldrich) and 1 mM phenylmethanesulfonyl fluoride and incubated on ice for 15 minutes. Cells were then lysed with a Dounce homogenizer, the nuclei were eliminated by centrifugation at 950 g for 3 minutes, and the lysate was desalted through a Sephadex G-25 column equilibrated in the lysis buffer. The assay was performed according to manufacturer’s recommendations in the presence or absence of camp analogs. Lysate samples (13 μg of protein) were incubated for 45 minutes at 25°C, and the reactions were stopped by heating at 95°C for 10 minutes. The catalytic C subunit provided in the kit was used as a positive control. Phosphorylated and nonphosphorylated kemptides were separated by gel electrophoresis, and their fluorescence under ultraviolet illumination was analyzed using a CCD camera system (ImageQuant LAS 4000; GE Healthcare, Piscataway, NJ). The PKI peptide derived from the selective inhibitor of PKA (Cheng et al., 1986) was added at 180 μM to assess the contribution of non-PKA activity to total phosphotransferase activity. Basal and stimulated PKA activity was quantitated as the PKI-inhibited fraction of the phosphorylated kemptide.

**Whole Cell Extracts and Western Blot Analysis.** Cells were washed twice with ice-cold PBS and lysed with 8% SDS, 0.25 M Tris pH 6.8, 1% protease inhibitor cocktail (P8340), 2.5 mM NaF, and 0.2 mM NaN3. The lysates were vortexed and boiled twice for 5 minutes. Insoluble material was removed by centrifugation at 18,000 g for 1 hour. The protein concentration was determined using a bicinchoninic acid protein assay (Thermo Scientific, Rockford, IL). Total cellular proteins were separated by SDS-PAGE following the technique described by Laemmli (1970). The cell extraction and electrophoresis conditions for two-dimensional gels were as previously described elsewhere (Wu et al., 2006).

Total cellular proteins separated by SDS-PAGE were transferred onto polyvinylidene fluoride membranes. The blots were blocked with 5% nonfat dry milk, 0.5% Tween 20 in PBS, pH 7.4 (PBS-Tween-milk), and then were probed with one of the following primary antibodies: anti-PKA-RIα, anti-PKA-RIβ, anti-PKA-Cα, anti-PKA-RIβ-P-S114, or anti-PKA-RIα-P-S99 (610609, 610625, 610980, 612550, or 552844, respectively; BD Biosciences, Franklin Lakes, NJ), anti-PKA-RIβ (539233; Merck Millipore, Darmstadt, Germany), anti-PKA-RIα (sc908; Santa Cruz Biotechnology, Santa Cruz, CA), anti- glyceraldehyde 3-phosphate dehydrogenase (ab4855; Abcam, Cambridge, UK), anti-actin (A2066; Sigma-Aldrich), or anti-vimentin (NCL-VIM-V9; Novocastra Laboratories, Newcastle, UK) diluted in PBS-Tween-milk. After washing, the blots were incubated with the appropriate peroxidase-conjugated secondary antibody diluted in PBS-Tween...
milk, washed again, and developed by chemiluminescence (Western Lighting Plus-ECL; Perkin Elmer, Waltham, MA). When indicated, protein dephosphorylation was performed using λ protein phosphatase (Merck Millipore). Dephosphorylation was performed by incubation of the blots at room temperature in 50 mM Tris pH 7.4, 150 mM NaCl containing 1% bovine serum albumin (BSA), 0.1% Triton X-100, 2 mM MnCl₂, and 400 U/ml λ protein phosphatase for 1 hour. The blots were then washed with 0.1% Tween-20 in PBS and processed as described previously for immunologic detection.

Conventional and Confocal Immunofluorescence Microscopy. The NB4 cells were fixed in 2% paraformaldehyde in PBS pH 7.4 for 10 minutes at room temperature, permeabilized with 0.1% Triton × 100 in PBS pH 7.4 for 10 minutes, and blocked in 2% BSA in PBS, pH 7.4 (PBS-BSA). The cells were incubated with the primary antibody diluted in PBS-BSA (610165, anti-PKA-RI; BD Biosciences; sc 908, anti-PKA-II; Santa Cruz Laboratories; 610625, anti-PKA-IIβ; BD Biosciences; or G6160, anti-β-catenin protein; Sigma-Aldrich) overnight at 4°C, washed in PBS, incubated with Alexa Fluor 488 or Alexa Fluor 594-conjugated secondary antibody (Life Technologies, Carlsbad, CA), diluted in PBS-BSA for 1 hour, and washed again in PBS. Cells were mounted on slides with 5 μl of mounting medium containing 4,6-diamidino-2-phenylindole (Dapi) (Vectorshied H-1200; Vector Laboratories, Burlingame, CA) to counterstain the nuclei. Cells were observed either by conventional fluorescence microscopy (Leica Microsystems Type R, equipped with 63× objective; Leica, Wetzlar, Germany) or laser scanning confocal microscopy (Zeiss LSM 510, equipped with 63× objective; Carl Zeiss, Oberkochen, Germany).

RNA Preparation and Quantitative Reverse-Transcription Polymerase Chain Reaction. Total cellular RNA was collected from the manufacturer using TRIzol reagent (Life Technologies) as described by the manufacturer. Reverse transcription using Transcriptor First Strand cDNA kit (Roche Diagnostics, Basel, Switzerland) and quantitative reverse-transcription polymerase chain reaction using the LightCycler cDNA kit (Roche Diagnostics, Basel, Switzerland) and quantitative PCR manufacturer. Reverse transcription using Transcriptor First Strand cDNA kit (Roche, Switzerland) and quantitative PCR manufacturer. RT-PCR amplification was performed in LightCycler and the LightCycler FastStart DNA MasterPLUS SYBR Green kit were performed as described previously elsewhere (Deville et al., 2011). PKA-R1β specific primers sequences were 5′-GGGAgTTTCCGGCAGGTCG-3′ for the sense primer located in exon 6 and 5′-CTCCTGAAGGTACCCTGTC-3′ for the antisense primer located exon 7. PKA-R1β mRNA level was normalized to the expression of the ribosomal protein P2 mRNA measured in parallel using a sense primer 5′-GACCC00CCTACAAAGOTAT-T-3′ and an antisense primer 5′-CCACCAAGTACCCAC-T-3′ located in exon 5 and exon 9–4, respectively.

Cell Maturation Assays. NB4 cells committed into the granulocytic differentiation pathway can be identified before morphologic evidence is observed by analyzing the expression of the RNA levels of the ribosomal protein P2 mRNA measured in parallel using a sense primer 5′-GACCC00CCTACAAAGOTAT-T-3′ and an antisense primer 5′-CCACCAAGTACCCAC-T-3′ located in exon 5 and exon 9–4, respectively.

Results

NB4-LR1 Cells Are Less Sensitive Than NB4 Cells to Cotreatment with 8-CPT-cAMP and ATRA. Previous studies have shown that the endogenous intracellular cAMP concentration is lower in maturation-resistant resting NB4-LR1 cells than in NB4 cells, and it has been proposed that this may contribute to their differential response to ATRA-induced maturation (Zhao et al., 2004). To investigate whether this lower cAMP level in NB4-LR1 cells fully explain their blunted ATRA responsiveness, we undertook a comparative NBT maturation assay of NB4 and NB4-LR1 cells treated with ATRA in combination with a maximally effective concentration (0.2 mM) of 8-CPT-cAMP. This potent exogenous cAMP analog, as expected (Queenec-Du et al., 1998), allowed robust maturation of NB4 cells at low ATRA concentrations, but was less efficient for the NB4-LR1 cells (Fig. 1, A and B). This indicates that the deficient ATRA responsiveness cannot be explained by low cAMP level only. We searched therefore for an additional defect in the cAMP/PKA signaling downstream of cAMP for a possible explanation of this behavior.

One explanation could be that the NB4-LR1 cells contained less PKA than the NB4 cells. Therefore, PKA activity was determined in both cell lines in basal and 8-CPT-cAMP stimulated conditions using a nonradioactive cAMP-dependent protein kinase assay based on the phosphorylation of a kemptide. As the kemptide could be phosphorylated by kinase other than PKA, the addition of a selective inhibitor (PKI peptide) was used as a means to determine the actual contribution of PKA activity. Under basal conditions (without 8-CPT-cAMP), the PKI sensitive portion of the kemptide phosphorylation was almost negligible in both NB4 and NB4-LR1 cells. Upon PKA stimulation (addition of 8-CPT-cAMP) the phosphorylation of the kemptide significantly increased and was completely inhibited by the addition of the PKI, demonstrating that the extract kinase activity was due mainly to PKA. When analyzed comparatively, no significant difference in PKA activity was observed between extracts from NB4 and NB4-LR1 cells in the presence of PKA-saturating concentration of 8-CPT-cAMP, even after ATRA treatment (Fig. 1C). The similar overall content of PKA was further demonstrated by immunoblotting of the catalytic (C) subunit of PKA, which showed similar level in NB4 wild-type and NB4-LR1 cells (Fig. 2A). We concluded that the blunted ATRA responsiveness of NB4-LR1 cells was not due to lower overall PKA activity.

ATRA-Resistant NB4-LR1 Cells Are Deficient in PKA Type II. We considered next whether NB4-LR1 cells differed from NB4 cells or NB4-LR2 cells with respect to PKA isofrom expression or localization. In NB4-LR2 cells, ATRA-maturation resistance is caused by a PML-RARα mutation (Duprez et al., 2000) without impairment of cAMP signaling. Immunoblot analysis showed that NB4 and NB4-LR2 cells expressed the RIα regulatory subunit of PKA-I and both the RIIα (50 kDa) and RIIβ (52 kDa) subunits of PKA-II (Fig. 2A). Note that the blots probed with the anti-PKA-RI/RIβ antibody revealed an additional faint 50 kDa band (pointed out by an asterisk in Fig. 2A). This band was demonstrated by two-dimensional gel electrophoresis to be RIIα (Fig. 2B), indicating that the anti-PKA-RI/RIβ antibody has a slight cross-reaction with RIIα. No RIIβ subunit was detected in any of the cell lines, using a specific anti-PKA-RIβ antibody (unpublished data).

The NB4-LR1 cell extracts showed no detectable PKA-RI/RIβ protein band and less PKA-RIα (Fig. 3A). The underexpression of PKA-RI/RIβ in NB4-LR1 compared with NB4 was reflected at the mRNA level in quantitative real-time polymerase chain reaction analysis using specific primers (Fig. 2C). Interestingly, NB4-LR1 cells expressed more PKA-RIα protein. Hence, the NB4-LR1 cells have a shift in isozyme composition, expressing more PKA-I and less PKA-II than NB4 and NB4-LR2 cells.
PKA-RIIβ protein was detected (Fig. 3), in agreement with the Western blot studies (Fig. 2). Altogether, the data demonstrated a deficient expression of PKA type II, compensated by an overexpression of PKA type I in the ATRA maturation-resistant NB4-LR1 cell line as compared with the ATRA-sensitive NB4 cell line and the NB4-LR2 subline. It could thus be hypothesized that the PKA isozyme switch from PKA-II to PKA-I could interfere with maturation of the NB4-LR1 cells.

**PKA-RII Is Not Induced by ATRA/cAMP Maturation Treatment in NB4-LR1 Cells.** Retinoids exert part of their antitumoral effects through reactivated expression of silenced genes (Villa et al., 2007; Abecassis et al., 2008). Therefore, we wondered whether ATRA/cAMP signaling cross-talk necessary for NB4-LR1 maturation could modulate the PKA-RII subunit expression. For this purpose, we determined the PKA regulatory subunit expressions in NB4-LR1 cells whose differentiation was triggered by a combination of ATRA (1 μM) and 8-CPT-cAMP (0.2 mM), a potent PKA-I and PKA-II activator. The increased actin expression and concomitantly decreased vimentin expression with a constant expression of glyceraldehyde 3-phosphate dehydrogenase (Fig. 4) confirmed immature NB4-LR1 leukemia blast differentiation toward neutrophilic granulocyte. We noted a slight increase in PKA-RIIα, but it never reached the level of expression observed in both NB4 and NB4-LR2 cells. Of note, PKA-RIα subunit expression was also slightly increased during this treatment, but the differentiation was not accompanied by PKA-RIIβ protein expression. Therefore, a high expression of the PKA-RIIβ isoform is not obligatory for the ATRA/cAMP signaling cross-talk leading to maturation of NB4-LR1 cells.

The subcellular localization of PKA-RI was qualitatively similar in the NB4 and NB4-LR1 cells, showing a diffuse slightly granular and mainly cytoplasmic distribution (Fig. 3). Although present at very low level throughout the cytoplasm, PKA-RIα was mainly distributed in a perinuclear region in either cell type, suggesting a Golgi localization (Fig. 3A). Double immunofluorescent labeling of PKA-RIα and the Golgi marker β-coatamer protein followed by confocal microscopy analyses confirmed this localization (Fig. 3B). In NB4 cells, the labeling pattern of PKA-RIIα was similar to that of PKA-RIα. A Golgi localization of PKA-RIIα and PKA-RIIβ has been also reported in other cell types (Rios et al., 1992; McCahill et al., 2005). In NB4-LR1 cells, the expression of PKA-RIα appeared slightly less intense compared with NB4 cells and little or no PKA-RIIβ was mainly detected (Fig. 3), in agreement with the Western blot studies (Fig. 2). Altogether, the data demonstrated a deficient expression of PKA type II, compensated by an overexpression of PKA type I in the ATRA maturation-resistant NB4-LR1 cell line as compared with the ATRA-sensitive NB4 cell line and the NB4-LR2 subline. It could thus be hypothesized that the PKA isozyme switch from PKA-II to PKA-I could interfere with maturation of the NB4-LR1 cells.

**Both PKA-I and PKA-II Must Be Activated to Achieve Maximal ATRA-Induced Differentiation of NB4 and NB4-LR1 Cells.** To evaluate the contribution of each PKA isozyme, we exploited the fact that certain cAMP analogs prefer one of the two cAMP binding sites (A, B) of either the RI

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**Fig. 1.** (A) Effect of strong PKA stimulation (0.2 mM 8-CPT-cAMP) on the NB4 and NB4-LR1 cell maturation response to various concentrations of ATRA. NB4 and NB4-LR1 cells were exposed to ATRA (0–16 nM) for 48 hours. Maturation was visualized by the NBT reduction assay. (B) Quantification of maturation in NB4 (white bars) and NB4-LR1 (black bars) cells exposed to ATRA in the presence of 0.2 mM 8-CPT-cAMP. Data are given as the mean ± S.D., n = 3. (C) In vitro PKA catalytic activity in NB4 and NB4-LR1 cells treated (+) or not (−) with 100 nM ATRA. Kinase activity was quantified in cell lysates by measuring the phosphorylation of the fluorescent kemptide in the absence or presence of 1 μM 8-CPT-cAMP (CPT). The addition of a highly specific PKA inhibitory peptide (PKI, 180 μM) was used as a means to determine the actual level of PKA activity.

**Fig. 2.** PKA subunits expression in NB4 cell lines. (A) Expression of PKA subunits (RIα, RIα, RIβb and C) analyzed by Western blot in NB4, NB4-LR1, and NB4-LR2 cells. Actin was used as a control. *An additional minor protein band corresponding to a cross-reaction of anti-PKA-RIIβ antibody with PKA-RIIIs subunit. (B) Two-dimensional Western blot analysis of PKA-RII subunits in NB4 and NB4-LR1. The region of the blot corresponding to protein sizes between 35 and 75 kDa was cut out and probed consecutively with specific PKA-RIIα, PKA-RIα, and vimentin antibodies (arrow). The lower panel presents the merged picture. (C) Expression of PKA-RIIβ mRNA in NB4 and NB4-LR1 cells was analyzed by quantitative reverse-transcription polymerase chain reaction, normalized to the expression of ribosomal P2 mRNA and expressed as a percentage of that detected in NB4 cells. Data are given as the mean ± S.D., n = 4.
or RII subunit of PKA (Supplemental Table 1) (Ogreid et al., 1989; Huseby et al., 2011). In one series of experiments performed on NB4-LR1 cells treated with 0.1 μM ATRA, the DCF maturation assay showed that 8-Pip-cAMP, which selects site BI of PKA-I, and the AII-directed N^6-MBC-cAMP (Fig. 6A). This result indicates that coactivation of both PKA-I and PKA-II is required to trigger the maturation response of NB4-LR1 cells. Therefore, the residual PKA-II in the NB4-LR1 cells had to be activated to achieve maturation.

We studied next the requirement of PKA-I and PKA-II in NB4 cell maturation. Because the pharmacologic concentration of ATRA alone induces NB4 cells maturation, the response of these cells to site-specific cAMP analogs can only be investigated at a lower ATRA concentration. Indeed, it has been previously shown that NB4 maturation can be induced at near physiologic concentrations (2–15 nM) of ATRA by either exogenous 8-CPT-cAMP treatment (Fig. 1) or a sustained increase in the endogenous level of cAMP produced by an autonomous bacterial adenylate cyclase (Quenech'Du et al., 1998). We observed (Fig. 5B) a significant synergistic effect on maturation only when combining cAMP analogs that complemented each other for binding to the cAMP sites (A, B) of both PKA-RI and PKA-RII, like 8-Pip-cAMP (AI, BII), 2-Cl-8-MA-cAMP (BI, BII), and N^6-MBC-cAMP (AII). This result indicates that coactivation of PKA-I and PKA-II is required to trigger the maturation response also of NB4 cells.

This was confirmed in a second series of experiments with other site-specific cAMP analogs using the NBT maturation assay. The site AI-selective N^6-Bz-8-piperidino-cAMP was combined with the site BI-prefering 2-Cl-8-AHA-cAMP to achieve preferential activation of PKA-I, and the site AII-preferring N^6-MCB-cAMP combined with the site BII-prefering 5,6-DiCl-eBIMPS to achieve PKA-II activation. We found no differentiating effect of either cAMP analog pair alone at 2 nM ATRA (Fig. 5C, left) and only a moderate effect at 15 nM ATRA (Fig. 5C, right). However, the PKA-I and PKA-II directed pairs acted with strong synergy to produce a similar level of maturation as obtained with 0.2 mM 8-CPT-cAMP (Fig. 5C). The synergizing effect of adding the PKA-II directed analog pair to the PKA-I pair was not due to the activation of Epac, another intracellular target of cAMP. Indeed, the Epac specific cAMP analog 8-pMeOPT-2′O-Me-cAMP alone was unable to promote ATRA-induced differentiation of NB4-LR1 contrary to N^6-benzoyl-cAMP, a poor Epac agonist and a full PKA activator (Supplemental Fig. 1). Furthermore, a strong synergy was observed also when the PKA-I pair was mixed with the site AII-directed analog N^6-MBC-cAMP (Fig. 5B), which does not activate Epac (Christensen et al., 2003).

The PKA-I and PKA-II directed cAMP analog pairs were tested for their ability to activate PKA in extracts prepared from either NB4 or NB4-LR1 cells. Figure 6 shows that the PKA in the NB4 cell extracts was only partially activated when the cAMP analog 8-Pip-cAMP was combined either with N^6-MBC-cAMP to achieve selective PKA-II activation or with 2-Cl-MA-cAMP to achieve PKA-I activation. Only the triple combination activating both PKA-I and PKA-II achieved near full activation. For extracts from the NB4-LR1 cells that contain more PKA-I and less PKA-II (see Figs. 2–4) the PKA-I directed cAMP analog pair was more efficient than the PKA-II directed analog pair, but again the triple combination gave the highest PKA activity. These results support the idea that both PKA-I and PKA-II activation are required to achieve the high PKA activation that appears required for optimal stimulation of ATRA-induced cell differentiation.

In NB4-LR1 Cells, PKA-RII Is Mainly in the Nonphospho Form at Basal cAMP Levels, but Becomes Autophosphorylated at High cAMP Concentration. The PKA-RII subunit, but not PKA-RI, can be autophosphorylated on a specific serine residue by the C subunit. The autophosphorylated RII subunit has about 10-fold decreased affinity for the C subunit of PKA, which leads to about 10 times higher sensitivity to activation by cAMP (Rangel-Aldao and Rosen, 1976, 1977). The PKA-RII autophosphorylation state in APL cells could therefore give an important clue about the cAMP responsiveness of their PKA-II. We determined therefore the...
extent of PKA-RII autophosphorylation in nonstimulated and 8-CPT-cAMP stimulated NB4-LR1 and NB4 cells by use of an anti-PKA-RIIα (pS99) phospho-directed antibody whose phospho-epitope specificity was confirmed by phosphatase treatment (unpublished data). A strong constitutive phosphorylation of PKA-RIIα was visible in untreated NB4 cells, and a slight increase was induced after treatment with 0.2 mM 8-CPT-cAMP combined or not with ATRA (Fig. 7). In contrast, PKA-RIIα phosphorylation was hardly detectable in untreated NB4-LR1 cells. However, and importantly, 8-CPT-cAMP treatment alone or combined with ATRA was able to reveal a significant enrichment of autophosphorylated PKA-RIIα. Note that even though the increase of phosphorylated PKA-RIIα could partly be related to the low increase in total RII levels in NB4-LR1 cells, the densitometric quantification of protein bands showed that in response to 8-CPT-cAMP the mean level of phosphorylated-RIIα normalized to total PKA-RIIα is 2.7-fold higher than in nontreated NB4-LR1 cells.

These results indicate that PKA-II is little autophosphorylated and thereby is subresponsive to cAMP in NB4-LR1 cells with a low cAMP level, but becomes more autophosphorylated and thereby more cAMP-responsive under strong stimulation of cAMP signaling.

**Discussion**

Our study has revealed an altered balance between PKA-I and PKA-II in the ATRA-maturation-resistant NB4-LR1 APL cell line, whose ATRA resistance has been previously ascribed to lowered adenylate cyclase activity and less intracellular cAMP (Zhao et al., 2004). We showed that NB4-LR1 cells had a deficient ATRA response compared with wild-type NB4 cells even when PKA was fully activated by a potent cAMP analog. In fact, these cells had a specific defect in PKA-RII that resulted from decreased expression of both PKA-RIIα and PKA-RIIβ isoforms. This decrease of RII subunits was compensated for by an increase of the RIIα subunit, with the level of the PKA-Cα and the global PKA activity being unchanged. The isozyme switch led to a translocation of PKA because PKA-RII was concentrated in the perinuclear...
Golgi area, whereas PKA-RI had a general cytoplasmic distribution (Fig. 3). It is known that distinct cellular responses to cAMP can be elicited upon selective activation of one isozyme, and that the cAMP response can be influenced by PKA isozyme switching (Schwartz and Rubin, 1985; Rohlff et al., 1993; Cho-Chung et al., 1995; Ji et al., 2008). By using isozyme-specific cAMP analogs, we demonstrated that the activation of the two PKA isoforms (PKA-I and PKA-II) pathways were required for retinoid dependent maturation of NB4-LR1 cells.

The analog combinations used in our study are the same as extensively validated for PKA-I and PKA-II selectivity in vitro (Ogreid et al., 1985), supplemented with second-generation analogs with further enhanced in vitro PKA isozyme specificity as well as deficient ability to activate Epac. The first generation analog combinations were validated for cell use in fibroblasts with enforced expression of either RI or RII subunit (Otten et al., 1991). The improvements of the second generation analog 2-Cl-8-AHA-cAMP are a further enhanced specificity for cAMP site B of PKA-I and a very low affinity for Epac (Dao et al., 2006). The second-generation analog N6-Bz-8-Pip-cAMP has both improved selectivity for site AI and a complete lack of ability to activate Epac due to its N6-substitution (Christensen et al., 2003).

These observations point to a specific key role of the PKA-II for maturation, even though PKA-I is upregulated probably due to a compensation mechanism. Therefore, PKA-I activation and PKA-II activation are essential to support the maturation of both NB4-LR1 and NB4 cells (Fig. 5).

These conclusions were supported by the in vitro assay showing the contribution of each PKA isozyme to the total PKA activity. Although a cAMP stimulation strong enough to activate both PKA-I and PKA-II will enhance the sensitivity of the NB4-LR1 cells to pharmacologic ATRA concentrations, it cannot overcome the intrinsic subsensitivity of these cells to low ATRA concentrations. In fact, NB4-LR1 cells remained maturation resistant at physiologic ATRA concentrations even at maximally stimulatory concentrations of cAMP agonists. However, even though PKA-I and PKA-II need to be activated, it remains to be established whether they need to be activated at the same time or consecutively. Several genes that appear to be regulated by ATRA may be under the control of the cAMP-PKA signaling pathway, which supports the idea of a complex signaling network. Therefore, it will be important to identify downstream targets of PKA-I/PKA-II coactivation.

Fig. 6. Activation of both PKA I and PKA II is required for full PKA activation. PKA activity was quantified in cell lysates by measuring the phosphorylation of the fluorescent kemptide in the absence (–) or presence of site-specific cAMP agonists selective for PKA-I and PKA-II. The relative PKA activity (measured as the PKI-sensitive portion of kemptide phosphorylation) was expressed as the percentage of the maximal activity measured when the cell lysates were incubated in the presence of 1 μM 8-CPT-cAMP. A near full PKA activation was reached only when both PKA-I and PKA-II were activated by the combination of the three site-specific cAMP analogs. Data are given as the mean ± S.E.M., n = 2–3.

Fig. 7. Activation of PKA-RIIα by 8-CPT-cAMP treatment. (A) NB4 and NB4-LR1 cells were cultured in the absence (–) or presence (+) of 1 μM ATRA and 0.2 mM 8-CPT-cAMP alone or in combination for 24 hours. Total PKA-RIIα and its serine 99 phosphorylated form were analyzed by Western blot using an anti-PKA-RIIα and an anti-PKA-RIIα (pS99) phospho-specific antibody, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a loading control. (B) Phosphorylated and total PKA-RIIα bands were quantified by densitometry and normalized to GAPDH band. The results are expressed as the percentage relative to the control of nontreated NB4 cells arbitrarily set to 100. Values are the mean of three independent experiments.
The requirement for PKA-II activation to achieve NB4-LR1 cell maturation (Fig. 5A) led us to study whether the juxtanuclear PKA-RII could be more sensitive to cAMP in the NB4-LR1 cells and thus could provide an efficient brake on PKA-dependent signaling unless exposed to a high level of cAMP or cAMP analogs. In tissues such as the heart, cAMP stimulation converts the PKA-RII from the more cAMP-sensitive autophosphorylated state to the relatively cAMP-insensitive dephosphorylated state (Manni et al., 2008), which will lead to a “negative feedback” of the cAMP-stimulated PKA activity (Kopperud et al., 2003). In contrast, in unstimulated NB4-LR1 cells, PKA-II was mainly in the nonautophosphorylated state, and it became strongly phosphorylated in cells exposed to the strong cAMP agonist 8-CPT-cAMP. The PKA-II will therefore exhibit a “feed-forward” response to increasing concentrations of cAMP agonists.

The low cAMP sensitivity of the nonphosphorylated PKA-II in the NB4-LR1 cells represents a challenge for the achievement of cAMP-stimulated maturation of NB4-LR1 cells and thus could provide an efficient brake on cell maturation in a clinical setting. Although PKA-I may be responsible for the recurrence of APL in some patients treated with ATRA, the definition of a strategy for ATRA/cAMP combinatorial therapies is considered to enhance ATRA-induced APL cell maturation in a clinical setting. Although PKA-I may be activated by mild stimulators of the endogenous cAMP level, PKA-II may be hard to activate without making use of PKA-II directed cAMP analogs. Our data should contribute to defining a molecular basis for the design of combinatorial therapy and encourage either the integration of PKA-II directed analogs or the development of agents directed at releasing PKA-RII from its A-kinase anchoring protein into in vivo testing in mice.

Authorship Contributions

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