Cyclin D1 is a NF-κB corepressor

María F. Rubia, Pablo N. Larrosa Fernandez a,1, Cecilia V. Alvarado a, L.C. PANELO a, Marina Ruiz Greccoa, Georgina P. Coloa, Giselle A. Martínez-Noela, Sabrina M. Micenmachera, Mónica A. Costasa,b,⁎

aLaboratorio de Biología Molecular y Apoptosis, Instituto de Investigaciones Médicas Alfredo Lanari (IDIM-CONICET), Universidad de Buenos Aires, Combatientes de Malvinas 3150, C1427ARO Buenos Aires, Argentina.
bArgentine National Research Council (CONICET), Argentina.

1 These authors contributed equally to this work.

ARTICLE INFO

Article history:
Received 8 June 2011
Received in revised form 27 December 2011
Accepted 17 January 2012
Available online 28 January 2012

Keywords:
Cyclin D1
NF-κB
RAC3
Transcriptional activity
Tumor development
Nuclear receptor coactivator

ABSTRACT

NF-κB regulates the expression of Cyclin D1 (CD1), while RAC3 is an NF-κB coactivator that enhances its transcriptional activity. In this work, we investigated the regulatory role of CD1 on NF-κB activity. We found that CD1 inhibits NF-κB transcriptional activity through a corepressor function that can be reverted by overexpressing RAC3. In both, tumoral and non-tumoral cells, the expression pattern of RAC3 and CD1 is regulated by the cell cycle, showing a gap between the maximal expression levels of each protein. The individual increase, by transfection, of either CD1 or RAC3 enhances cell proliferation. However the simultaneous and constitutive over-expression of both proteins has an inhibitory effect. Our results suggest that the relative amounts of CD1 and RAC3, and the timing of expression of these oncogenes could tilt the balance of tumor cell proliferation in response to external signals.

© 2012 Published by Elsevier B.V.

1. Introduction

NF-κB is a transcription factor formed by protein dimmers containing the Rel dimerization domain, where Rel-A (p65)/p50 heterocomplex is the best characterized and plays a pivotal role in the regulation of diverse biological processes, including immune response, development, cell growth and survival [1,2]. NF-κB can be rapidly and transiently activated by a large variety of stimuli, such as DNA damage, cytokines, microbial components and mitogens [3], and induces a variety of genes involved in cell proliferation and survival, including Cyclin D1 (CD1) [4,5]. Gene transactivation, induced by NF-κB, is regulated by a dynamic balance between the activity of coactivators and corepressors [4,7]. The deregulated function of NF-κB contributes to the development of a variety of human diseases, particularly immune-related diseases and cancers [8–10].

In human breast cancer, NF-κB stimulates tumor cell proliferation and blocks programmed cell death [11,12]. Particularly, the transcriptionally active form of this factor was detected in estrogen receptor (ER)-negative tumors and has been linked to tumor development [13]. Moreover, the activation of NF-κB is also required for estrogen-induced proliferation in ER-positive tumor cells [14]. Such effects of estrogens are mediated by a complex that contains the activated ER, the nuclear receptor coactivator RAC3 and a member of the NF-κB family that induces CD1 expression through the binding to a conserved sequence that contains kB elements in the promoter of the gene [4,5].

RAC3 is a member of the Steroid Receptor Coactivator (SRC) family that enhances the transcriptional activity of several nuclear receptors and NF-κB [6,15–17]. The molecular mechanism by which coactivators induce chromatin remodeling and transcriptional activation involves a histone acetyltransferase (HAT) activity, present in the C-terminal domain of SRC-1 and RAC3 [18,19], and the recruitment of other general coactivators such as CBP/p300, p/CAF, CARM-1 and PRMT1 [20,21].

On the other hand, CD1 is considered an oncogene with an important tumorigenic role in breast cancer and other human tumors [22]. Emerging evidence suggests that CD1 may act through novel pathways that do not involve its widely accepted function on the cell cycle. Instead, it may also exhibit such novel activities independently of its function as Cyclin-dependent kinase (Cdk) regulatory subunit [23–25]. It has been suggested that CD1 may activate ER-α-mediated transcription through a direct interaction with the ligand-binding domain of the receptor, and the recruitment of the SRC coactivators [26]. A similar mechanism, but with opposite effect, has been reported for the Androgen Receptor (AR), where CD1 has an inhibitory role [27]. In this case, the mechanism appears to partially depend on both

Abbreviations: CD1, Cyclin D1; Cdk, Cyclin-dependent kinase; ER, Estrogen receptor; HAT, Histone acetylase; HDAC, Histone deacetylase; PMA, 12-O-tetradecanoylphorbol-13-acetate; SRC, Steroid receptor coactivator; RAC3, Retinoic acid coactivator
⁎ Corresponding author at: Laboratorio de Biología Molecular y Apoptosis, Instituto de Investigaciones Médicas Alfredo Lanari (IDIM-CONICET), Universidad de Buenos Aires, Combatientes de Malvinas 3150, C1427ARO Buenos Aires, Argentina. Fax: +54 11 4523 8947.
E-mail address: mcostas@lanari.fmed.uba.ar (M.A. Costas).
1 These authors contributed equally to this work.
Histone deacetylase (HDAC) recruitment and competition for AR coactivators, such as p/CAF [28]. In agreement with these observations, over-expression of CD1 inhibits the activation of the Thyroid Receptor (TR) in both ligand-dependent and independent manners, by recruiting HDAC3 [29].

We and others have previously demonstrated that NF-κB activity is regulated by coactivators that usually bind to nuclear receptors [6,15–17]. On the other hand, although CD1 is a target gene for NF-κB, it has the ability of recruiting coactivators and corepressors. These evidences suggest the existence of a complex regulatory network involved in the control of the cell cycle-dependent gene expression.

Thus, in this work we investigated the role of CD1 as a regulator of NF-κB activity. We found that CD1 physically interacts with NF-κB and inhibits its transactivation in an HDAC-dependent way. Our results support the existence of an unexpected antagonistic relationship between CD1 and RAC3, usually over-expressed in tumors. These results have important implications for the understanding of the regulatory networks that affect the progression of cancer.

2. Materials and methods

2.1. Cell culture and reagents

The human breast tumor T47D cells were grown in Dulbecco’s modified Eagle’s F12 medium (DMEM-F12) and the human embryonic kidney HEK293 cells were maintained in DMEM (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO2. Unless stated otherwise, all reagents were obtained from Sigma Chemical Co. or Santa Cruz Biotechnology, USA. For all the experiments the relative densitometric units (RDU) were determined from the original gel or picture using the NIH-Image J software.

2.2. Expression vectors and reporter plasmids

The reporter plasmid containing the consensus sequence for binding of NF-κB (κB-Luc) and plasmid containing the CD1 promoter upstream
of Luciferase (PromCD1-Luc) were previously described [6,14]. The expression vector for pCMX-RAC3 was a gift from Dr. R. Evans, The Salk Institute, San Diego, USA. Dr. Eduardo Canepa, Universidad de Buenos Aires, Argentina kindly provided bacterial expression vectors encoding Cyclin D1. The full-length Cyclin D1 gene was cloned into pcDNA6/V5-His ABC (Invitrogen) at the Bam H1 and Eco R1 sites.

2.3. Immunoprecipitation and Western blot analysis

Co-immunoprecipitation and Western blot were performed as previously described [14]. Briefly, for immunoprecipitation assays, after 45 min of incubation with 10 ng/ml of phorbol ester 12-0-tetraecanoylphorbol-13-acetate (PMA), T47D or HEK293 cells were scraped and treated with RIPA buffer containing aprotinin, peptatin A, phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). Protein extracts were incubated overnight at 4 °C with anti-

2.4. Luciferase assays

HEK293 cells were plated in 24-well plates 24 h prior to transfection at a density of 250,000 cells/well. Cells were transiently transfected with a total of 0.2 μg of DNA (including pcDNA6-CD1 or pCMX-RAC3 or pSilencer-CD1-siRNA plus 20 ng of κB-Luc plasmid or 20 ng of PromCD1-Luc and 5 ng RSV-βGal) using the calcium phosphate precipitation method. The medium was replaced after 5 h and cells were stimulated with 10 ng/ml of PMA or 20 ng/ml of human TNF-α 2 h later.

The assays for luciferase and β-galactosidase activity were performed after 6 h of treatment using the appropriate substrates following the manufacturer’s protocols (Promega Corp.). To achieve transfections with a constant amount of DNA, appropriate amounts of the parental empty expression vector (pcDNA6/V5-His ABC or pCMX) were added to each well. In some experiments, cells were pre-incubated with deacetylases inhibitor Trichostatin A (TSA) for 30 min before stimulation with PMA.

For assays of Cyclin D1-promotor activity, HEK293 cells were transfected with the reporter plasmid PromCD1-Luc plus pcDNA6-CD1, the sshκB expression vector or the empty vector together with RSV-βGal. The assays for luciferase and β-galactosidase activity were performed after 24 h of treatment with PMA (10 ng/ml) using the appropriate substrates following the manufacturer’s protocols (Promega Corp.).

2.5. Electrophoretic mobility shift assay (EMSA)

HEK293 cells transiently transfected with expression vector for CD1 24 h, were then stimulated with 10 ng/ml of PMA for 60 or 180 min and EMSA was performed as previously described [14]. Briefly, for each assay, 5 μg of nuclear extracts were incubated with 1 ng of κB probe, containing the sequence 5'-AGTTGAGGGGACTTTCC-3'.
CAGGC-3′, and 200 μg of poly dIdC for 15 min at room temperature. For competition assays, 5–20 fold excess of the unlabeled κB oligonucleotide was utilized. Complexes were separated in non-denaturing 5% PAGE. Gels were dried under vacuum and autoradiographed at −70 °C.

2.6. Chromatin immunoprecipitation (ChIP)

After incubation for different time periods with 20 ng/ml of TNF-α, T47D or HEK293 cells were fixed with 1% of formaldehyde for 10 min and the ChIP was performed as described previously [14]. Immunoprecipitation was performed using 25 μg of DNA in RIPA buffer with anti-Rel-A, anti-CD1, anti-RAC3 or anti-HDAC1 antibodies for 18 h at 4 °C in an orbital rocker. Immunoprecipitates were then incubated with GammaBind Sepharose-protein G (GE) for 2 h at 4 °C in an orbital rocker. After three washes, DNA was eluted from the pellets, and proteins were digested with Proteinase K. Samples were heated with shaking at 65 °C for 4 h to reverse crosslinking. The extract containing DNA was precipitated with ethanol 70% and sodium acetate 0.3 M. Samples were resuspended in buffer TE and PCR was performed using the primers 5′-TTCATTCAGGCTTGTTGTTCTC′ and 5′-GGATTCACGGTTAGCATGCG-3′ that correspond to the distal −749 and −858 κB elements of the CD1 promoter [4,14]. The κB-α primers were designed to cover the NF-κB binding site (−352/+1) in the human κB-α gene promoter [31] forward: 5′-GGATTCAGCGACGAC-3′ and reverse: 5′-CCCTA TAAACGCTGGCTG-3′ and performing 35 cycles.

2.7. Proliferation assays

HEK293 cells transiently transfected with the expression vectors for CD1, RAC3, both simultaneously or the empty vectors, were

Fig. 2. Effect of cyclin D1 on NF-κB nuclear localization and DNA-binding activity. A. HEK293 cells were transfected with 0.1 μg of a CD1 expression vector, or with an empty vector. After 24 h cells were stimulated, during 1 or 3 h, with PMA (10 ng/ml). Nuclear extracts were prepared and analyzed by EMSA using double-strain oligonucleotides containing the NF-κB binding site. B. The specific binding was determined by competition with unlabeled κB oligonucleotide. C. Nuclear extracts were analyzed by Western blot and probed with antibody against Rel-A, CD1 or Histone 3.
plated in 96-well flat bottom plates at a density of 8000 cells/well in 100 μl of medium. After 24 h, medium was replaced with serum-free medium and 16 h later, cells were released to the cell cycle by addition of medium containing 10% FBS. Cells were fixed at specific time points after medium change and the proliferation was determined by staining with 0.5% crystal violet. Absorbance of surviving stained cells was measured at 570 nm.

2.8. Soft-agar colony formation assay

HEK293 cells were transfected as described above. Twenty-four hours after transfection, cells were detached from the plates with trypsin. Cells were resuspended as individuals in DMEM growth medium with the full supplements described above mixed at a 2:1 ratio with agarose 0.7%. The mixture was then plated onto six-well plates at 5×10⁴ cells/well over a bottom layer of 0.5% agarose in DMEM with the supplements. Cells were maintained at 37 °C with a medium change every 3 days. Four weeks later, cell aggregates with diameters of 0.2 mm or larger (containing approximately 50 or more cells) were counted as colonies. The entire experiment was performed twice.

2.9. Real time PCR

Total mRNA was isolated from HEK293 cells by using the TRIZOL protocol (Invitrogen). Reverse transcription was carried out by using the SuperScript II kit (Invitrogen) per the manufacturer’s instructions. For gene expression analysis, qPCR was performed by using sequence-specific primers for: Vimentin forward 5'-GAACCTGAGGGAACCTAATCTG-3' and reverse 5'-CTGAGAAGGTCCGTA-TACC-3', RAC3 forward 5'-AAGTGAAGGGATCTGGA-3' and reverse 5'-CAGATTGACTACCTGAGG-3' and GFP forward 5'-ACCATCTTCTTGAACCTGAGG-3'.

---

Fig. 3. Repression of NF-κB through CD1 involves deacetylases and is reversed by RAC3 over-expression. A. HEK293 cells were co-transfected with NF-κB-Luc reporter plasmid plus RSV-β-Gal in the presence or absence of increasing amounts of CD1 expression vector. Cells were stimulated for 30 min with 30 nM TSA or DMSO, prior to treatment with PMA 10 ng/ml for 6 h. B. Co-expression of increasing amounts of RAC3 expression vector, plus an expression vector for CD1 (0.15 μg). Relative luminiscence units (RLU) are the average of triplicate ± SD, and were normalized with the corresponding β-galactosidase values. C. RAC3 mRNA expression normalized to the mRNA expression of GFP and GAPDH. * p<0.001 with respect to the values without CD1 over-expression. **, *** p<0.001 with respect to the same amount of CD1 expression vector but without TSA. # p<0.001 with respect to the values without RAC3 over-expression. ##, ###, #### p<0.001 with respect to the same amount of RAC3 expression vector without CD1 expression vector. Analyzed by Tukey’s Test.
GGACGA-3′ and 5′-GGCTGTTTAGTTGTACTCC-3′. For all analyses, GADPH forward 5′-TCTCCTGACTCAACAGCC-3′ and reverse 5′-GTTGTCATACCCAGAAATGA-3′ was used as an internal control.

3. Results

3.1. Cyclin D1 plays an inhibitory role over NF-κB transcriptional activity

Since NF-κB activity is affected by some of the molecules that bind to CD1, we therefore hypothesized that this cyclin might affect the transcriptional activity of NF-κB. In order to verify this, HEK293 cells were co-transfected with a κB-Luc reporter plasmid in the presence or absence of different mass (0.1, 0.15 and 0.2 μg) of a vector encoding for CD1 or an empty vector, and the control β-Gal plasmid. Transfected cells were stimulated with 10 ng/ml of PMA and Luciferase activity was measured after 6 h. We found that increased levels of CD1 expression vector inhibited NF-κB activity (Fig. 1A ~40% inhibition, bars 2 vs 4). The levels of CD1 after transfection with the CD1 expression vector are shown in Fig. 1B.

In order to determine if this inhibitory effect is mediated by a specific action of CD1, we performed additional experiments of κB-Luc reporter assays where the HEK293 cells were also transfected with an expression vector for the siRNA of CD1 that clearly inhibits CD1 expression (Fig. 1C). As shown in Fig. 1D, the inhibition of endogenous CD1 significantly increases the NF-κB transactivation in basal or stimulated conditions and blocks the inhibitory effect of the CD1 expression vector.

A similar CD1 effect was observed when NF-κB was activated using 20 ng/ml of TNF-α, an inflammatory cytokine that exerts several physiological actions and is a powerful inducer of the canonical NF-κB pathway [32,33]. Fig. 1E shows an inhibition of 45% for 0.1 μg of CD1 expression vector respect to the empty vector transfected cells.

In order to determine the effect of CD1 over an endogenous NF-κB target gene, we analyzed if the expression of Vimentin is modified by changes in the levels of CD1. HEK293 cells were transfected with different mass of CD1 expression vector and then stimulated with PMA during 18 h. The levels of Vimentin and its mRNA were analyzed by Western blot and Real time PCR. Fig. 1F and G shows that the increase of CD1 inhibits the expression of Vimentin in agreement with the results obtained on κB-Luc reporter assays.

3.2. Cyclin D1 does not inhibit the NF-κB nuclear translocation

NF-κB dimmers are sequestered in the cytoplasm as latent complexes through binding to members of a family of IκB proteins [1]. Because CD1 has an inhibitory effect on NF-κB transcriptional activity when activation is induced by both PMA and TNF-α, it shows to be independent of the activation pathway. Thus, we first hypothesized that CD1 might inhibit the nuclear translocation of NF-κB or the DNA-binding ability of the transcription factor. Afterwards, the levels of nuclear active NF-κB were analyzed by EMSA. In these experiments,
HEK293 cells were transfected with the CD1 expression vector or an empty vector and NF-κB activation was induced by PMA stimulation. As shown in Fig. 2A, the increased levels of CD1 did not inhibit the amount of active NF-κB available in the nucleus that could bind the specific oligonucleotide (Fig. 2A), indicating that CD1 does not antagonize with NF-κB activation.

The composition of these complexes was confirmed by performing super-shift experiments in the presence of antibodies against p50 or Rel-A subunit of NF-κB (Fig. 2B compare lanes 2, 3, and 4). In addition, the specificity of binding was determined by competition assays using the specific unlabeled κB oligonucleotide (Fig. 2B compare lanes 5, 6, and 7). The composition of the complexes is indicated in the figure.

The absence of CD1 inhibition of NF-κB nuclear translocation after 3 h of PMA stimulation was also confirmed by Western blot experiments, performed with the nuclear proteins from each experimental condition (Fig. 2C). Thus, these results demonstrate that CD1 does not inhibit NF-κB nuclear translocation or its ability to bind DNA. Moreover, the effect of CD1 over NF-κB activity involves a mechanism that is subsequent to nuclear translocation and DNA binding.

3.3. The repression of NF-κB transactivation by CD1 involves a deacetylase activity

It has been previously demonstrated that CD1 inhibits the activity of TR and AR, through HDAC recruitment [28, 29]. In order to investigate whether the CD1-mediated repression of NF-κB involves a deacetylase activity, we analyzed the effect of 30 nM TSA over the κB-Luciferase activity in presence of different levels of CD1. As shown in Fig. 3A, the transactivation of NF-κB, induced by PMA, increased 22-fold with respect to the basal conditions, while this activation was inhibited by CD1 over-expression (~40% inhibition; bar 4 with respect to bar 2). However, treatment with TSA inhibits the repressive action of CD1 in cells transfected with low levels of this expression vector (bar 12 with respect to bar 4), indicating that deacetylases are involved in the CD1 transrepression of NF-κB.

Although the absence of a more significant effect of TSA under high levels of CD1 could be suggesting additional CD1 repressive mechanisms, our results are in agreement with previous observations concerning the HDAC recruitment by CD1 and demonstrate that these molecules are involved in CD1 inhibition of NF-κB.

3.4. The NF-κB activity is regulated by the levels of CD1 and RAC3 in an antagonistic way

RAC3 is a NF-κB coactivator that has and recruits HAT activity [6, 34]. In view of our results, concerning the participation of HDACs in the CD1 mediated transrepression of NF-κB, we hypothesized that increased levels of RAC3 could revert the effect of CD1 by increasing the NF-κB activity.

Therefore, we performed κB-Luc reporter assays in order to analyze the activity of NF-κB, in HEK293 cells co-transfected with increasing mass of the RAC3 expression vector, with or without a constant mass of CD1 expression vector. Fig. 3B shows that high levels of RAC3, as expected, increase the activity of NF-κB, while high levels of CD1 inhibit the NF-κB coactivator role of RAC3. Interestingly, under the highest mass of RAC3 together with high CD1 expression (bar 12) the values are similar to that obtained in the absence of both expression vectors (bar 2).

These studies indicate that RAC3 and CD1 antagonize in their function over the NF-κB activity, which is sensitive to the relative amounts of these proteins.

3.5. Cyclin D1 regulates transcriptional activity of NF-κB as part of a protein complex that binds to DNA at NF-κB binding sites

The abundance of CD1 affects local histone acetylation and methylation of specific promoters, as detected in chromatin immunoprecipitation assays [35]. Therefore, it is possible that CD1 could be regulating the NF-κB activity by altering local chromatin structure, through the recruitment of corepressors over the promoters of target genes.

Since CD1 expression inhibits the activity of NF-κB, and this was partially reversed by deacetylase inhibitor or over-expression of RAC3, we decided to investigate if CD1 and NF-κB may be co-associated in the context of the local chromatin structure of an NF-κB target gene. We used a chromatin immunoprecipitation assay to analyze the binding of Rel-A, RAC3, HDAC1 and CD1 to a DNA fragment of CD1 promoter containing κB binding elements [14]. These experiments were performed in HEK293 and T47D cells and NF-κB was activated by TNF-α treatment (Fig. 4A and B respectively). The recruitment of these molecules was monitored during a time frame of 15–180 min after stimulation.

Fig. 4A and B shows that Rel-A/DNA interaction is increased at 30 or 15 min respectively and reduced at 60 minutes post TNF-α
stabilization in both cell lines. Interestingly, we detected the CD1 association with its own promoter even in the absence of TNF-α treatment; however, its recruitment was increased after stimulation, peaked at 15 min and gradually reduced after 30 min. This result suggests that CD1 can be a player in the regulation of NF-κB transactivation, perhaps acting like a corepressor assisting in the recruitment of HDAC.

RAC3 was examined in the same experimental conditions. Interestingly, when we compared the patterns of RAC3/DNA association between HEK293 cells and T47D cells (Fig. 4A versus B), we observed that the kinetic of DNA association was different. While in HEK293 cell line RAC3 association was increased at 15 min and reduced after 30 min of stimulation, in T47D the RAC3 recruitment peaked at 15 min and remained associated after 180 min of TNF-α stimulation.

Concerning HDAC1 recruitment to CD1 promoter, we could observe in both cell lines a particular kinetics: it was associated to DNA in the absence of treatment similar to that observed for CD1 and reduced after 30 min of TNF-α stimulation, just when Rel-A/DNA association peaked. Moreover, while in HEK293 cell line HDAC binding was increased again at 60 min, in T47D cells re-association occurs after 180 min of stimulation.

These results demonstrate that CD1 exerts an inhibitory effect over NF-κB, acting at the promoter level, where the transcription factor binds and follows a kinetic association that is synchronized to the recruitment of other coregulators that regulate the on/off for the NF-κB target gene.

In order to determine whether HDAC1 association to the NF-κB target promoter depends on the CD1 recruitment, a similar experimental model was performed in HEK293 cells, but using the CD1 siRNA expression vector. Fig. 4A shows that inhibition of the endogenous CD1 correlates with a lower recruitment of HDAC1 to the promoter. Similar results were obtained for RAC3 recruitment under conditions of Rel-A inhibition (Fig. 4A, right panel).

Because CD1 is a NF-κB target gene, we also analyzed the effect of CD1 over-expression over its own promoter as a functional correlate of these associations and its corepressor action. HEK293 cells were co-transfected with a PromCD1-Luc reporter plasmid, the expression vector for CD1, or the empty control in the presence or the absence of the NF-κB inhibitor ssIκB expression vector. The reporter activity was determined after PMA stimulation. Fig. 4C shows that high levels of CD1 expression inhibit the NF-κB-induced transcription from the CD1 promoter.

In order to determine if recruitment of CD1 to an NF-κB target promoter is specific for the CD1 promoter, we performed ChIP assays analyzing a known target for NF-κB: the IκB promoter [31]. As shown in Fig. 4D, CD1 is also recruited to this promoter.

3.6. Cyclin D1 and the transcription factor NF-κB could be part of the same protein complex

In agreement with the obtained results, CD1 could be inhibiting NF-κB transactivation at level of their target DNA sequence, suggesting that at least for a short time after NF-κB stimulation, both proteins could be part of the same protein complex. Therefore, we investigated if CD1 could be found associated to NF-κB proteins. In these experiments T47D human breast tumor cells were stimulated with PMA in order to activate NF-κB, and physical interaction was analyzed by co-immunoprecipitation.

Fig. 5A shows that Rel-A was detected in the CD1 immunoprecipitate, while CD1 was detected in the Rel-A immunoprecipitate, indicating that this member of the NF-κB family and CD1 could be part of the same protein complex.

Since T47D cell line over-expresses CD1, at similar levels to those reported previously for human breast cancer [36], we decided to analyze these interactions using an additional cell line with normal levels of CD1, like the human embryonic kidney HEK293 cells. As shown in Fig. 5B, results were similar to that observed in T47D cells.

In addition, we also analyzed if Cdk4, the kinase partner of CD1, could be part of this complex. Fig. 5A and B, shows, as expected, that CD1 interacts with Cdk4, however this kinase is not part of Rel-A containing complexes.

These results indicate that NF-κB and CD1 could be part of the same protein complex, not depending on CD1 over-expression, and independent of Cdk4 binding.

3.7. CD1 and RAC3 showed different cell cycle expression pattern and their co-expression at high levels inhibits proliferation and anchorage-independent growth

In order to determine the CD1 and RAC3 expression profile, HEK293 and T47D cell lines were synchronized for 16 hours serum starvation.

As shown in Fig. 6A and B, both cell lines show a similar temporarily gap between the CD1 and RAC3 expression profiles. While CD1 peaked 3 h after serum addition, RAC3 expression was increased 3 h later in both cell lines. These results suggest that the expression of both proteins is synchronized following particular kinetics, perhaps in agreement with their role in the cell cycle.

Since NF-κB is regulated by CD1 and RAC3, with antagonistic effects, we also analyzed the basal NF-κB activity in HEK293 cells, by reporter assays, at the same time that CD1 and RAC3 expression levels were measured. As shown in Fig. 6C, maximum activity of NF-κB coincides with minimal CD1 expression levels, showing a profile that is in agreement with the expected CD1 and RAC3 roles on NF-κB activity.

In order to analyze if CD1 and/or RAC3 over-expression may affect the cell proliferation, HEK293 cells were transfected with expression vectors for CD1, RAC3 or both together, and then cells were synchronized for 16 h by serum starvation. Cell proliferation was evaluated after 0, 12 and 24 h of serum addition, by staining with crystal violet.

As expected, CD1 over-expression induced proliferation (Figure 6D). The same results were obtained when RAC3 was over-expressed. Moreover, high levels of RAC3 could bypass the absence of growth factors promoting cell proliferation. However, the results obtained when both RAC3 and CD1 were simultaneously over-expressed, were particularly surprising because we observed a marked inhibition of cell proliferation, indicating there is an antagonism over the proliferative signaling induced by each one of these molecules when both are co-expressed at high levels and at the same time.

It has been previously demonstrated that RAC3 has a transforming role [37]; therefore, we also analyzed its transforming role and the effect of CD1 over-expression in the non tumoral HEK293 cells, that are naturally unable to growth in soft agar. As shown in Fig. 6E, cells transfected with an expression vector for the coactivator acquire the ability to form colonies in soft agar, similar to that observed in the tumoral T47D cell line. However, HEK293 with a simultaneous and constitutive RAC3 and CD1 over-expression are unable to form colonies, showing a phenotype similar to non tumoral cells (Fig. 6E).

4. Discussion

Aberrant RAC3 and CD1 expression [38,39] has been linked to multiple types of human cancer and tumoral progression, and both proteins have an important role in the control of cell proliferation [40–42]. The present work describes a new role for Cyclin D1 as a NF-κB corepressor.

It is well documented that CD1 has several effects that are independent to its role in the cell cycle, acting as a coactivator or corepressor [26–29]. This cyclin strongly inhibits ligand-dependent AR activation [27] and it has been recently suggested that may compete with p/CAF coactivator for AR binding, and that the excess of coactivator expression may abrogate the repressor function of CD1 [28]. In
agreement with this observation, we demonstrate that CD1-mediated repression is partially reversed when a coactivator with intrinsic HAT activity is over-expressed, and it depends on deacetylase activity.

In response to cellular cues there is a turnover in the nuclear or cytoplasmatic localization of CD1 and NF-κB. Specifically, NF-κB translocates to the nucleus after IκB phosphorylation followed by proteasomal degradation [2]. Although it could be possible that CD1 inhibited NF-κB activity through cytoplasmic sequestration or blocking the DNA-binding activity of the transcription factor, we demonstrated that over-expression of CD1 did not prevent NF-κB nuclear translocation or its ability to bind DNA.

There are several studies showing transcriptional repression mediated by CD1 and involving the recruitment of HDAC activity [28,29]. In this work we examined the ability of the deacetylase inhibitor TSA to relieve CD1-mediated repression, and we observed that this inhibitor partially reversed the inhibitory action of CD1 over NF-κB. These data indicate that deacetylation is involved in the CD1 corepressor function probably through the recruitment of HDAC molecule to the NF-κB complex. Alternatively, it is possible that CD1 action is dependent on deacetylase activity but independent of histones. For example, it has been shown that Rel-A subunit can be acetylated by p300 [43], and Rel-A deacetylation by HDAC3 increases the interaction with IκB-α and the cytoplasm localization of the complex [44]. However, in our experiments no changes on nuclear Rel-A levels were found under PMA stimulation and increased expression of CD1, suggesting that this is not the mechanism by which CD1 inhibits NF-κB activity, at least under PMA treatment.

We found that the inhibition of endogenous CD1 by siRNA increases NF-κB activity, in a similar way that the increase found under PMA treatment. These results are in agreement with the inhibitory role for CD1 over NF-κB activity, even under physiological levels of expression for both molecules. Moreover, the ChIP assays demonstrate that CD1 could be part of a protein complex bound to κB elements that correlates with increased HDAC1 recruitment. Afterwards, inhibition of endogenous CD1 could be increasing the basal transcriptional activity of this transcription factor.

Because the binding of coactivators to transcription factors in the chromatin context is able to displace corepressor complex, we analyzed if RAC3 over-expression could reverse CD1-mediated repression. Our results shown, as expected, that increased levels of RAC3 enhanced NF-κB transactivation and partially reverted the CD1 trans-repression. Moreover, there is an antagonistic effect between CD1 and RAC3 over NF-κB transactivation that depends of the relative levels of these proteins.

In ER-negative human breast cancer, constitutive NF-κB activity is required for tumoral progression and cell proliferation [13,45].

Fig. 5. Cyclin D1 and the transcription factor NF-κB are part of the same protein complex. Whole cells extracts from A. T47D or B. HEK293 cells stimulated with PMA (10 ng/ml) for 45 minutes, were immunoprecipitated (IP) with CD1, Rel-A or Cdk4 antibodies. The immunoprecipitates were analyzed by Western blot with the same antibodies. NS corresponds to not immune serum immunoprecipitation.

\[ A \]

<table>
<thead>
<tr>
<th>PMA (10 ng/ml)</th>
<th>α-Rel-A</th>
<th>α-CD1</th>
<th>α-Cdk4</th>
<th>whole extracts</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

\[ B \]

<table>
<thead>
<tr>
<th>PMA (10 ng/ml)</th>
<th>α-Rel-A</th>
<th>α-CD1</th>
<th>α-Cdk4</th>
<th>whole extracts</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
have previously demonstrated that ER-α and NF-κB could be part of the same protein complex and this may induce CD1 expression [14]. The fact that CD1 expression and CD1/Cdk4 kinase activity are required for cell cycle progression [41], reveals a paradox on NF-κB-dependent proliferation: while CD1 is a target gene for NF-κB [46], it could also exert an anti-mitogenic function independent of Cdk4, inhibiting NF-κB activity as a feedback mechanism.

It has been previously shown that during cell cycle progression, the transition from G1 to S phase occurs with reduced levels of CD1 [4,5]. Among other mechanisms, this diminish effect could be the result of CD1 repression over NF-κB activity. This is consistent with the results of ChIP assays, where CD1 is able to bind κB-sequences, of its own promoter, in unstimulated conditions and may also recruit HDAC1. However, when NF-κB activity was stimulated, we observed a displacement of corepressor complex and a recruitment of RAC3. These observations suggest a model where the activation of NF-κB induces CD1 expression and cell cycle progression, but under high levels, CD1, could bind to its own promoter and recruit corepressor

![Fig. 6](image)
complexes silencing NF-κB activity. The kinetics of complex associations should be probably dependent of co-regulator levels and additional regulatory signals. Herein, we demonstrated that the simultaneous CD1 and RAC3 over-expression has profound anti-proliferative effects, underscoring the importance of relative CD1 and RAC3 levels on tumors. RAC3 is linked to cancer because it was found often amplified and/or over-expressed in several tumors [38,47–49]. Previous works showed no correlation between RAC3 and CD1 amplification, suggesting that these events may represent two different types of breast tumors [47]. Then, what happens to cells that over-express both of these molecules? Our results suggest that despite the deregulation in the expression levels, the cells still retain its cell cycle-dependent regulation. RAC3 expression is required for both normal and malignant cells to effectively enter S phase [42]. Moreover, our results indicate that RAC3 expression appears to be cell cycle-regulated both in the normal and malignant cells. Despite these studies, little is known about how elevated levels of RAC3 and CD1 may induce tumorigenesis and/or promote tumor growth. Interestingly, we observed that CD1 and RAC3 kinetics of expression was similar between both cell lines, despite the differences concerning endogenous levels and tumoral capabilities. In agreement with these observations and our own results, it can be suggested that with a physiological context, the antagonism observed between RAC3 and CD1 on the NF-κB transactivation can be compensated by a constitutive activation of NF-κB expression [50]. However, it is interesting to remark that in our experiments of over-expression, both molecules are under control of constitutive promoters, which are not the natural targets for cell cycle control. Therefore, the normal kinetic of RAC3 and CD1 expression suggests that this kinetic could be a key element in avoiding cell cycle inhibition.

In summary, in this work, we demonstrated for the first time that CD1 can inhibit NF-κB activity, that it could be attributed to an NF-κB target promoter and antagonize the RAC3 coactivator function (Fig. 7). We conclude then that CD1 or RAC3 over-expression is a pro-tumorigenic and pro-neoplastic role that contributes to tumor development. Additionally, a particular kinetic in the control of the relative expression levels of both molecules is required for tumor promotion.
Fig. 7. Relative amounts of CD1 and RAC3 play a role in transforming and the balance of tumor cell proliferation. The levels of CD1 and RAC3 expression oscillate in the cell cycle. Although high expression of one of them is associated to tumor development and both have a role in the control of cell cycle, CD1 acts as a NF-κB co-repressor while RAC3 is a coactivator. Some NF-κB target genes that are involved in tumor development could be controlled in an opposite way by the recruitment of these molecules to their promoters.

References


