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14. ABSTRACT
The small GTP-binding protein Rac controls essential functions, including actin cytoskeleton reorganization, cell proliferation, cell cycle progression, adhesion, migration and invasion. The relationship of Rac to prostate carcinogenesis has not been extensively studied. However upstream activators of Rac have been described to be hyperactivated in prostate cancer, and it is well known that growth factors are very important in the control of prostate cancer proliferation and progression, as well as in the maintenance of growth during androgen independency. Chimaerins, through their Rac-GAP activity, accelerate the hydrolysis of GTP from Rac, leading to its inactivation. To date four chimaerin isoforms have been isolated and reported: α1, α2-, β1- and β2-Chimaerin. While α1- and β1-chimaerin are restricted to brain and testis, respectively, α2- and β2-chimaerin are widely expressed. No experimental information has been reported about the possible role of chimaerins in prostate cancer. Likewise, there are no information available about the expression of different chimaerin in prostate cancer cell lines. Our work hypothesis is that by inhibiting Rac function in prostate cancer cells, chimaerins will impair proliferation and reduce the invasive properties of prostate cancer cells.

15. SUBJECT TERMS
Rac; Rac-GAP; Chimaerin

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INTRODUCTION

The small GTP-binding protein Rac controls essential functions, including actin cytoskeleton reorganization, cell proliferation, cell cycle progression, adhesion, migration and invasion. Whereas the relationship of Rac to prostate carcinogenesis has not been extensively studied, several papers have described that upstream activators of Rac are hyperactivated in prostate cancer, and it is well known that growth factors are very important in the control of prostate cancer proliferation and progression, as well as in the maintenance of growth during androgen independency. Rac belongs to the Rho family of small GTP-binding proteins, and cycles between an "on" (GTP-bound) and an "off" (GDP-bound) state, steps that involve guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), which accelerate GTP hydrolysis, respectively (1-3)

Chimaerins, through their Rac-GAP activity, accelerate the hydrolysis of GTP from Rac1, leading to its inactivation. To date four chimaerin isoforms have been isolated: α1, α2-, β1- and β2-chimaerin. While α1- and β1-chimaerin are restricted to brain and testis, respectively, α2- and β2-chimaerin are widely expressed (4,5). However, there are no reported information or experimental data describing chimaerin activity and/or expression in prostate cancer cells. Since chimaerins inhibit Rac function, it is predictable that they will have profound effects on Rac mediated signaling, and therefore impact on the proliferative and invasive capacity of prostate cancer cells.

BODY

1- Characterization of chimaerin isozymes in prostate cancer cells

To determine which chimaerin isoforms are expressed in prostate cancer cells, we initiated studies to analyze their expression using non-quantitative RT-PCR. We generated isoform-specific primers for individual chimaerin isoforms and for GADPH, a housekeeping gene based on the reported human sequences. We used LNCaP and C4 prostate cancer cells as androgen-dependent models with different sensitivities to androgens. The PC3 cell line was used in our experiments as a model for androgen-independency cells. Our experimental data using non-quantitative RT-PCR (Figure 1) revealed that LNCaP cells do not express α2- or β2-chimaerin. C4 cells express only β2-chimaerin at very low levels, and PC3 cells express α2- and β2-chimaerin at similar levels.

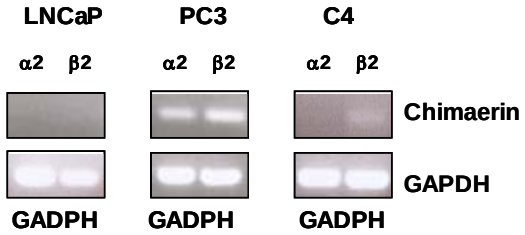

Figure1. Non-quantitative RT-PCR for α2-, β2-chimaerin and GADPH in LNCaP, PC3 and C4 cells.

In our laboratory, we have developed an anti-β2-chimaerin antibody (rat monoclonal) wich unfortunately was not highly specific, as it cross-reacts with α2-chimaerin (Figure 2A). Therefore we designed a peptide based on a β2-chimaerin specific sequence as well as a peptide for a α2-chimaerin specific sequence. These peptides were inoculated into rabbits in order to generate polyclonal antibodies. Different bleedings were obtained and subsequently tested by Western blot. Determination of chimaerin expression by Western blot with specific antibodies for each isoform was unsuccessful because unfortunately both antibodies showed cross-reaction (Figure 2B and C). It is not clear why this happened, as specific peptides were used. As the generation of specific antibodies is very important for this work as well as for the work of other members in our laboratory, we are currently generating new antibodies based on other peptide sequences.

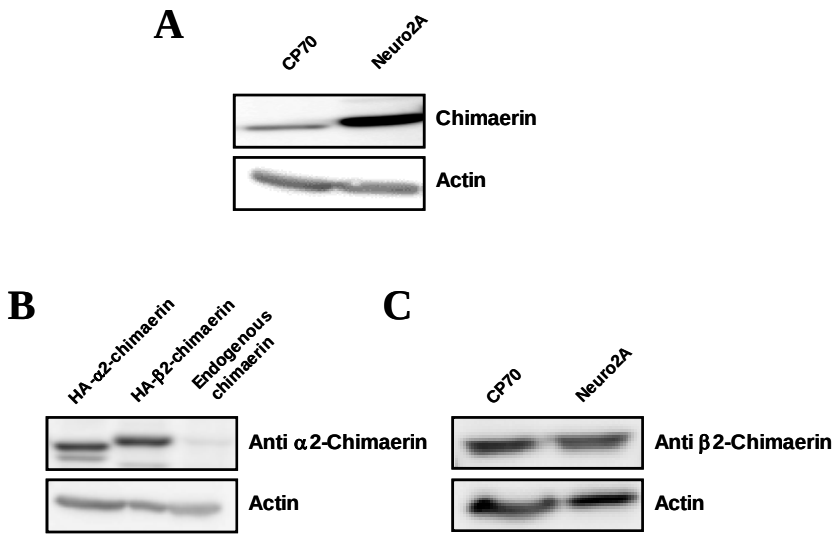

Figure 2. Analysis of chimaerin isoform-specific antibodies by Western blot. (A and C) Control cell lysates from the human ovarian cancer cell line CP70, that express α2- but not β2-chimaerin and murine Neuro 2A cell line, that expresses both isoforms. (B) Cell lysates from the human ovarian cancer cell line HeLa infected with α2- and β2-chimaerin AdV.

2- Cloning of α2 chimaerin

As a cDNA for the α2-chimaerin isoform was not available in our laboratory, and this tool would be needed not only for functional experiments but also for the characterization of the antibody, we decided to clone this cDNA using RT-PCR. We first generated cDNA from fresh PC3 mRNA, wich was used for RT-PCR using specific primers. Restriction sites were included in order to facilitate subcloning into various expression vectors. We have been successful in cloning this cDNA and in subcloning it into TOPO®- vector (Invitrogen). The identity of the cDNA was then confirmed by sequencing. Results revealed 100% identity with the sequence reported in NCBI databases, suggesting that no mutations have not been introduced in the PCR reaction.

3- Generation of chimaerin adenoviruses

The generation of recombinant adenoviruses to study the role of specific chimaerins represents an essential step to assess their cellular functions, as we have developed conditions in our laboratory to achieve nearly ~100% of the prostate cancer cells expressing recombinant proteins using adenoviral techniques. We used the AdEasy system (Stratagene, La Jolla, CA) to generate chimaerin adenoviruses. The multicloning site of this vector was modified to facilitate easy subcloning, and a N-terminal HA-tag has been included for easy detection. Previously, in our laboratory we have successfully generated adenoviruses for *wild type* β2-chimaerin (β2-chim-AdV) and the β2-chimaerin GAP domain (β-GAP-AdV).

I have been actively involved in the generation of other adenoviruses that would be needed in further experiments, as follows:

3a- β2 chimaerin

AdVs for GAP inactive mutants: **β2-chim-ΔEIE-AdV** and **β2-chim-298RRR-AdV**.
AdVs for phorbol ester/DAG unresponsive chimaerins: **β2-chim-C246A-AdV**.
AdVs for SH2 domain : **β2-chim-ΔSH2-AdV**.

The β2-chimaerin crystal structure of β2-chimaerin has been recently reported, as part of a collaborative effort between our laboratory and thelaboratory of Dr. Jim Hurley at the NIH (6). In this study it was found that several β2-chimaerin mutants present high sensitivity to phorbol ester-induced translocation as well as enhanced RacGAP activity *in vivo*. Due to this important finding we decided to incorporate these mutants to our study, as they will greatly contribute to our functional studies. Thus, I began the generation of adenoviruses encoding for two hyperactive mutants: **β2-chimaerin Q32A** and **β2-chimaerin -I130A**

3b- α 2 chimaerin

Using the AdEasy system I generated an adenovirus for *wild type* α2-chimaerin (α2-chim-AdV) and we are currently generating hiperactive mutants: **α2-chimaerin Q24A** and **α2-chimaerin I122A**, and the inactive mutant **α2-chimaerin P216A.**

Determination of Rac-GTP levels in prostate cancer cell lines

In order to determine whether Rac hyperactivation is observed in prostate cancer cells, Rac-GTP levels were determined in the presence of two serum concentrations (2% and 10%). This is important to detect constitutive Rac hyperactivation under limiting conditions of stimulation by growth factors. Rac-GTP levels were determined using a Rac-GTP pull down assay. This is a non-radioactive methodology based on the specific binding of Rac-GTP (but not Rac-GDP) to PBD (the PAK1 p21Rac binding domain) coupled to GST using glutathione-Sepharose 4B beads. After the pull-down samples were analyzed by Western blot with an anti-Rac1 antibody, and expression is normalized to total Rac levels in the corresponding cell extracts. Our experimental data (Figure 3) showed that Rac-GTP levels in LNCaP and C4 cells are similar in both, low and high serum,

whereas the androgen independent PC3 cells showed higher levels of Rac-GTP in 10% serum compared to cells growing in 2% serum. This data suggest that the androgen-dependent cell lines LNCaP and C4 probably have constitutive Rac activation in the absence of growth factors or mitogenic stimuli.

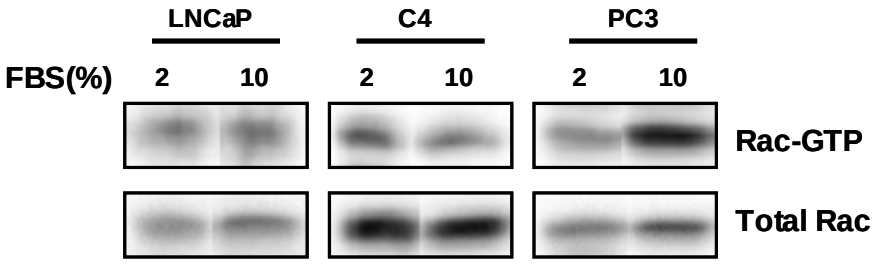

Figure 3. LNCaP and C4 androgen-dependent prostate cancer cells shows high levels of activate Rac at low serum concentrations. Human prostate cancer cell lines LNCaP, C4 and PC3 were grown in the presence of low serum (2%) or normal serum (10%). Active Rac (Rac-GTP) were determined using a pull-down assay.

5- Determination of Rac-GTP levels upon chimaerin AdV infection

LNCaP cells do not express α2- or β2-chimaerin, and C4 cells express only β2-chimaerin at very low levels, therefore we decided to use these model to assess if wether chimaerin overexpression by adenoviral delivery reduces Rac-GTP levels. LNCaP and C4 cells were infected with HA-tagged α2- and β2-chimaerin AdVs at different MOIs (25, 50 and 100 pfu/cell) and cultured in the presence of 2% or 10%l serum. Rac-GTP levels were determined 48 h after infection. We used a LacZ AdV as a negative control at 100 MOI. Experimental data (Figure 4) show that β2-chimaerin overexpression reduces Rac-GTP levels in LNCaP and C4 cells both in 2% and 10% serum, whereas α2-chimaerin only reduces Rac-GTP levels in the presence of 2% serum. This data suggest that β2-chimaerin may reduce the levels of hyperactive Rac both in the absence or presence of mitogenic factors, whereas α2-chimaerin only in conditions of low serum.. Further analysis will be performed to determine the mechanisms that explain the differences in sensitivity to either chimaerin isoform.

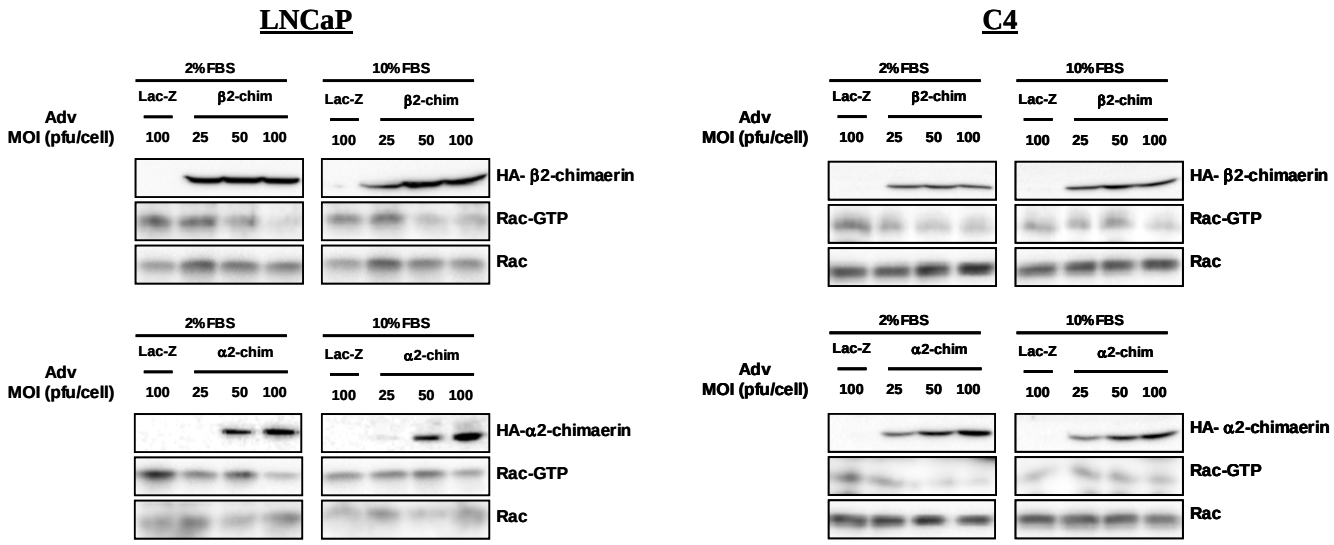

Figure 4. Chimaerin overexpression reduces Rac-GTP levels in LNCaP and C4 androgen-dependent prostate cancer cells. Human prostate cancer cell lines LNCaP and C4 were infected for 16 h with α2-, β2-chimaerin or LacZ AdV at different multiplicities of infections (MOIs) and grown in the presence of either 2% or 10% serum. Active Rac (Rac-GTP) was determined 48 h later using a pull-down assay.

6- Evaluation of MAPK signaling

Activation of Rac plays an important role in the control of the different MAPK signaling cascades. Since α2- and β2-chimaerin reduces Rac-GTP levels in LNCaP and C4 cells, we decided to study the effect of chimaerin overexpression on the activation of the various MAPK signaling pathways (ERK, JNK and p38 MAPK). LNCaP and C4 cells were infected with HA-tagged α2- and β2-chimaerin AdVs at different MOI (25, 50 and 100 pfu/cell) and cultured in the presence of medium with either 2% and 10% serum. Cell lysates were prepared 48 h after infection and subjected to Western blot analysis. Levels of activated ERK, JNK and p38 MAPK and the corresponding total levels were determined specific commercial antibodies. Experimental data shows that overexpression of β2-chimaerin (Figure 5, A, B) reduces the levels of activated ERK at MOI=50 pfu/cell both in the presence of 2% and 10% serum, but on the other hand, it induces a robust activation of p38 both in LNCaP and C4 cells. α2-chimaerin (Figure 5, C, D) reduces activated ERK levels only at high MOI (100 pfu/cell) and it reduces the levels of activated p38. No effect of chimaerin AdVs was found on JNK (data not shown). These data suggest a diferential regulation of MAPK cascades by chimaerin isoforms, an interesting finding that may translate into a differential regulation of genes and functional responses by either Rac-GAP. We are currently exploring the mechanisms involved in this differential effect of chimaerins isoforms.

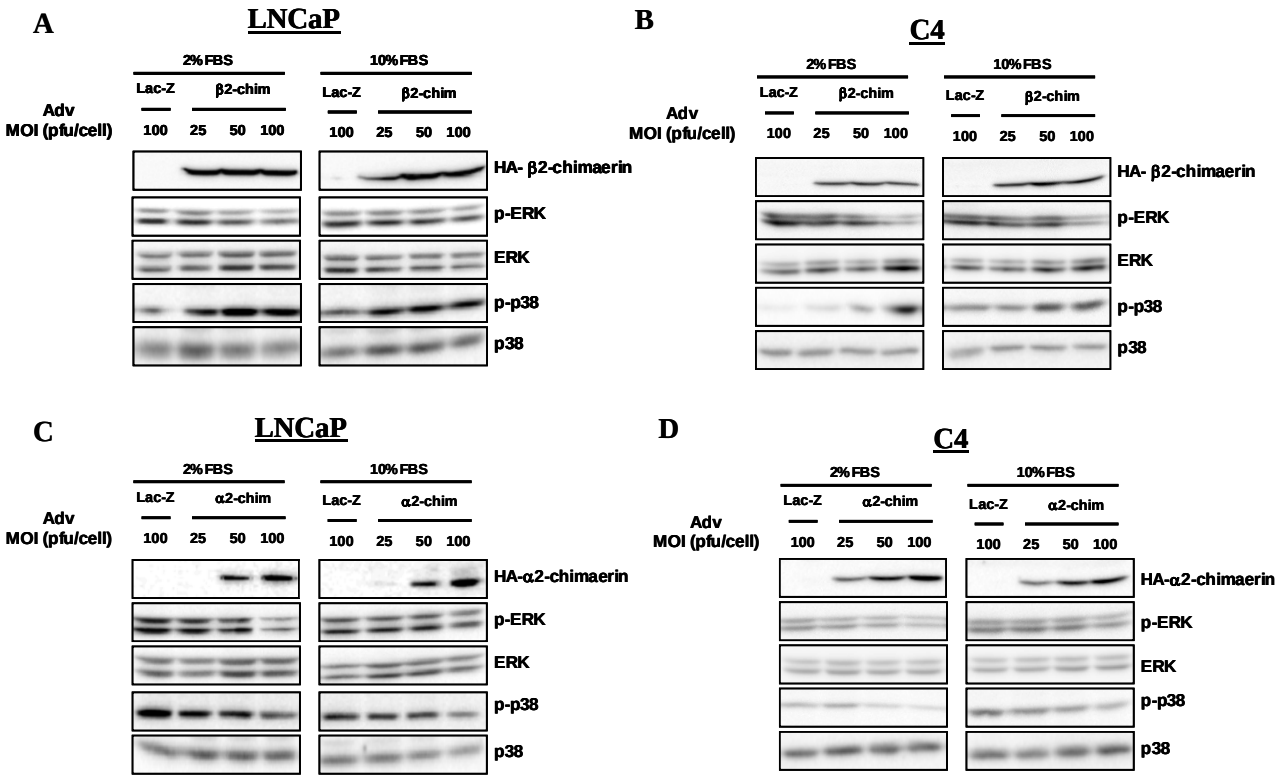

Figure 5. Effect of chimaerin isoforms on ERK and p38 phosphorylation. Human LNCaP and C4 prostate cancer cell lines were infected for 16 h with α2-, β2-chimaerin or LacZ AdV at different MOIs and grown in the presence of either 2% or 10% serum. P-ERK, ERK, p-p38 and p38 levels were determined 48 h later.

7- Studies on prostate cancer cell proliferation

Rac is required for proliferation and cell cycle progression (9,10,11). Our original hypothesis was that Rac inhibition by chimaerins impairs mitogenic responses, particularly in models of Rac hyperactivation. The fact that LNCaP and C4 cells shown Rac hyperactivation and that overexpression of α2- and β2-chimaerins reduces Rac activity and ERK activation was indeed strongly suggestive that chimaerins must impair cell proliferation. To addres this issue LNCaP and C4 cells were infected with HA-tagged α2- and β2-chimaerin AdVs at different MOIs (1, 5 and 50 pfu/cell) and cultured in the presence of 10% serum. Proliferation was assessed at 72 h after infection using an MTT assay. Experimental results (Figure 6) shows that only β2-chimaerin reduces cell proliferation (~ 25% reduction relative to non-infected cells). A control using LacZ AdV did not have any significant effect.

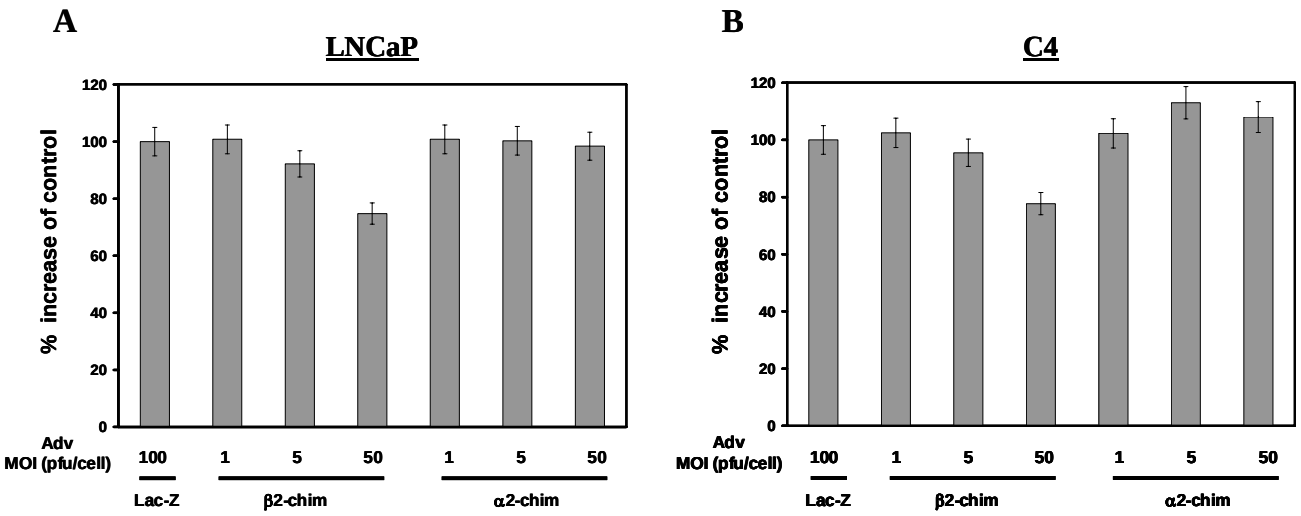

Figure 6. β2-chimaerin overexpression inhibits LNCaP and C4 cell proliferation. Human LNCaP and C4 prostate cancer cell lines were infected for 16 h with α2-, β2-chimaerin or LacZ AdV at different MOIs. MTT assay was performed 72 h later.

8- Cloning of a putative β2-chimaerin promoter

Transcription in eukaryotic cells is regulated at multiple levels. It has been determined that the methylation status of CpG islands in genes affects DNA-protein interactions. Most of the CpG islands in the promoter remain unmethylated, but when methylation occurs the gene is silenced. Thus, DNA methylation acts as a major epigenetic modification to maintain stable gene silencing. Aberrant DNA methylation impacts on gene transcription, and this event has been linked to cancer formation (7,8). Since several prostate cancer cell lines have low chimaerin expression, we sought to investigate whether the effect was at the promoter level. To date, there is no information on the β2 chimaerin promoter. We therefore decided to pursue this studies. Analysis of the gene using established programs reveal a region in the β2-chimaerin locus with promoter characteristics. Interestingly this region contains a putative CpG island. A 1 kb genomic DNA fragment 5´ upstream from the β2-chimaerin start codon was cloned by PCR into TOPO®- vector (Invitrogen) using specific primers.

Using PCR we have amplified and subcloned different fragments (see list above) of the 1 kb genomic segment into pRTK-Luc a plasmid that encodes for a luciferase gene under the control of a TK promoter. The constructs generated were as follows: **pR-81-Luc**, **pR-181-Luc**, **pR-281-Luc**, **pR-381-Luc**, **pR-481-Luc**, **pR-581-Luc**, **pR-1000-Luc** (the number represent the position of the first nucleotide upstream of the ATG). **pR-BASIC** (luciferase with no promoter) was used as a negative control and **pRTK-Luc** was used as a positive control.

These luciferase reporters were co-tranfected together with a Renilla expression vector (for normalization) into several cell lines. Experimental data revealed that all constructs induce luciferase expression upon transfection (Figure 7). We are currently analyzing in a comparative manner the various constructs upon transfection into LNCaP prostate cancer cells by using a new

system to delivery DNA into the cell that we have recently purchased (Nucleofector™ II). This is important to overcome the problem of lower efficiently of transfection in LNCaP cells.

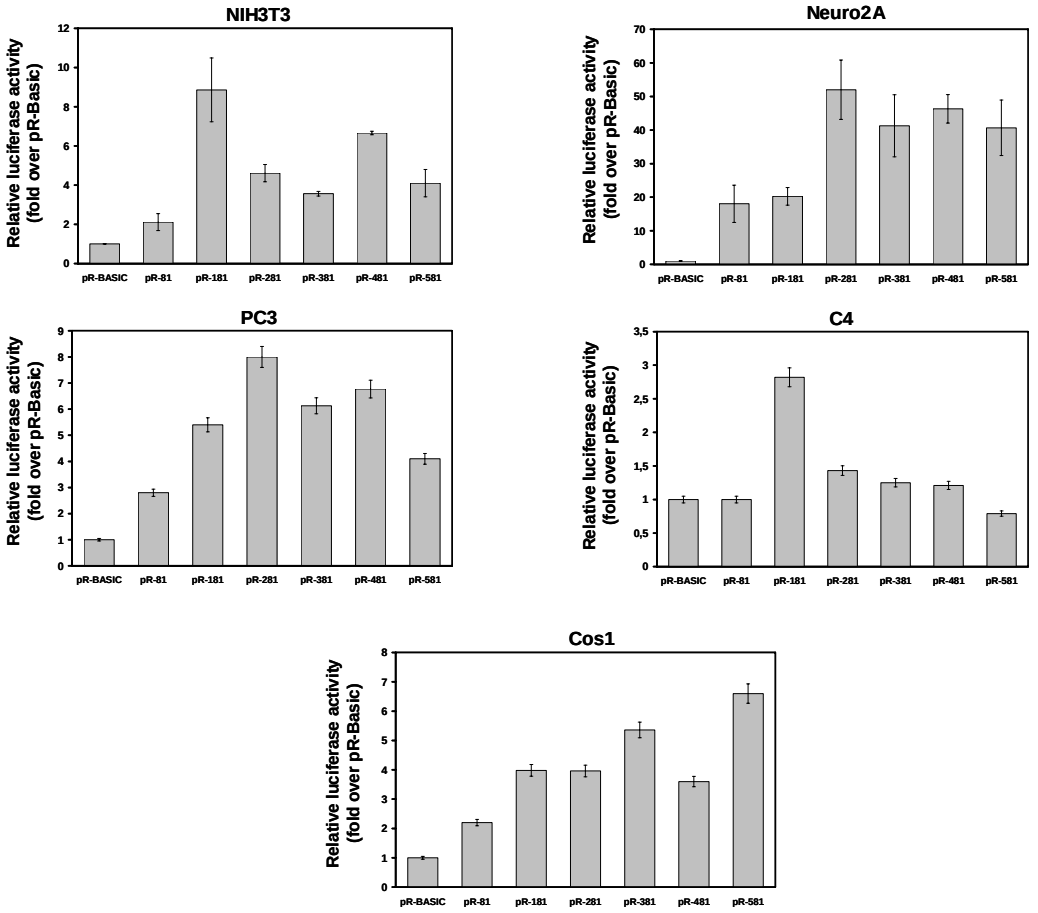

Figure 7. Luciferase activity of different constructs of β2-chimaerin putative promoter in several cell lines. NIH3T3, Neuro2A, PC3, C4 and Cos1 cells were transfected with 0.5μg of each reporter construct and Renilla, and 24 h after transfection luciferase activity of cell lysates was determined. Values represent the means ± S.D. from 3 different experiments.

In a different set of experiments we have focused on the methylation of the promoter. The constructs described above were methylated *in vitro* by using the enzymes SssI methylase, HpaII methylase and HhaI methylase. The methylation status was determined by digestion with the respectives enzymes and visualization in a agarose gel (Figure 8a). In a preliminary experiment we observed that upon transfection of the methylated constructs no luciferase activity could be detected (Figure 8b). These results suggest that the 1 kb fragment that we have isolated is indeed susceptible to methylation and that this modification influences the expression of the gene in certain cancer cells, including prostate cancer cells.

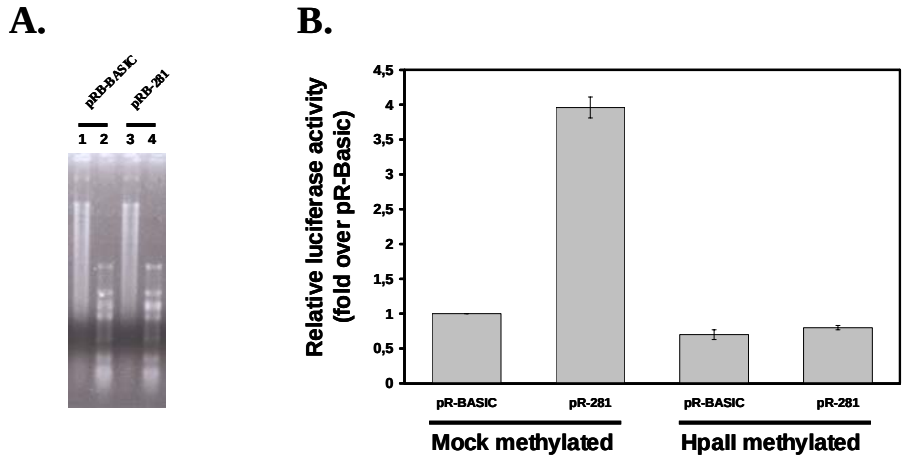

Figure 8. Effect of in vitro methylation on β2-chimaerin putative promoter activity. A, pR-BASIC and pRB-281 were methylated *in vitro* with HpaII methylase. The extent of methylation was assessed by comparing digestion patterns of unmethylated (lanes 2-4) and methylated (lanes 1-3) constructs with HpaII. **B**, methylated and unmethylated pR-BASIC and pR-281 constructs were transfected into Cos1 cells and assayed for luciferase activity. Values represent the means ± S.D. from 3 different experiments.

Future directions.

The next goals will be:

1. To assess wether chimaerins regulates ERK and p38 signaling pathways in prostate cancer cells.
2. To determine how chimaerins affect to cytoskeleton reorganization, migration and invasion in prostate cancer cells (Aim 3 from proposal). We will uso the Nucleofector™ II, a new DNA delivery system, to transfect GFP-chimaerin in LNCaP and C4 cells. The adenoviruses for different chimaerin isoforms and mutants will be used in addition in these experiments. These experiments were part of Specific Aim 3 in the original proposal, but we could not complete them in time. It is expected that these experiments will be done in the next few months.
3. To continue the characterization of the putative β2-chimaerin promoter and to determine whether methylation is a critical factor in β2 chimaerin expression.

KEY RESEARCH ACCOMPLISHEMENTS

1. We characterize the expression of chimaerin isoforms in prostate cancer cell lines.
2. We generated adenoviruses or adenoviral constructs that are essential for functional studies in prostate cancer cells.
3. We determined that Rac is hyperactive in some prostate cancer cell lines and that this hyperactivation could be reverted by chimaerin overexpression.

4. We determined that ERK activation levels could be reduced by chimaerin overexpression.
5. We determined that α2- and β2-chimaerin exerts opposites effects on p38 activation.
6. We cloned a 1 kb fragment that corresponds to the β2-chimaerin promoter.
7. We generated reporter constructs for different regions of the β2-chimaerin promoter, which will be used in luciferase assays.
8. We initiated studies aimed at determining whether the β2-chimaerin promoter is regulated by methylation.

REPORTABLE OUTCOMES

A manuscript is currently in preparation.

CONCLUSIONS

In the last year we completed experiments aimed at characterizing the regulation and function of β2-chimaerin and the related α2-chimaerin isoforms in prostate cancer cells. These proteins regulate the function of the small GTP-binding protein Rac, which plays essential roles in mitogenesis, transformation, and the metastatic cascade. We have obtained data regarding the expression of chimaerin isozymes in prostate cancer cell lines. We found that expression is very low in some prostate cancer cells, which is in agreement with data in other types of cancers, such breast cancer cells or gliomas. In addition, these prostate cancer cell lines presents high levels of activated Rac a situation that can be reversed by overexpression of chimaerins, wich also leads to reduce ERK activation and proliferation rate. The only divergence that we found between α2- and β2-chimaerin effects in prostate cancer cells is the different behavior on p38 phosphorylation. This is indeed the first evidence that probably α2- and β2-chimaerin may be having differential effect. The mechanisms have yet to be characterized.

The efficiency of transfection in LNCaP and C4 cells with usual DNA delivery systems is very poor (~5%). Since transfection in this cell lines is not efficient data about spreading and cytoskeleton reorganization were not consistent. Because that, at the present time we are using a new cDNA delivery system, Nucleofector™ II with provide a 80-90% of transfection efficiency. The development of the different adenoviruses will also help in this endeavour.

Understanding the events that control the expression of the β2-chimaerin gene is very important because the gene seems to be down-regulated in a variety of cancers. It would be important in the future to compare the expression of chimaerin in normal *vs.* cancer specimens. The cloning of a putative promoter and its characterization will further enhance the understanding of the regulation of this molecule.

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APPENDICES

None.

ABBREVIATIONS

AdV: adenovirus
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
DAG: diacylglycerol
GAP: GTPase activating protein
Kb: Kilobase
MOI: Multiplicity of infection
NCBI: National Center for Biotechnology Information
PKC: protein kinase C
PMA: Phorbol 12-myristate 13-acetate
PCR: Polymerase Chain Reaction
Rac-GAP: Rac-GTPase activating protein
TK: Thymidine Kinase