Functional Characterization of PRKAR1A Mutations Reveals a Unique Molecular Mechanism Causing Acrodysostosis but Multiple Mechanisms Causing Carney Complex

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Background : Mutations in the cAMP binding domains of the regulatory subunit of PKA (PRKAR1A) can cause either acrodysostosis or Carney-complex.

Results: All mutations impaired cAMP binding, but those resulting in Carney-complex had additional effects that increased PKA activity.

Conclusion: A single mechanism explains acrodysostosis, whereas diverse mechanisms cause Carney-complex.

Significance: First functional characterization of a series of PRKAR1A missense mutations causing acrodysostosis

ABSTRACT

The main target of cAMP is protein kinase A (PKA), the main regulatory subunit of which (PRKAR1A) presents mutations in two genetic disorders: Acrodysostosis and Carney complex. In addition to the initial recurrent mutation (Arg368X) of PRKAR1A gene, several missense and nonsense mutations have been recently observed in acrodysostosis with hormonal resistance. These mutations are located in one of the two cAMP binding domains of the protein and their functional characterization is presented here. Expression of each of the PRKAR1A mutants results in a reduction of forskolin-induced PKA activation (measured by a reporter assay) and an impaired ability of cAMP to dissociate PRKAR1A from the catalytic PKA subunits by BRET assay. Modeling studies and sensitivity to cAMP analogs specific for domain A (8-PIP-cAMP) or domain B (8-AHA-cAMP) indicate that the mutations...
impair cAMP binding locally in the domain containing the mutation. Interestingly two of these mutations affect amino acids for which alternative amino acid substitutions have been reported to cause the Carney complex phenotype. To decipher the molecular mechanism through which homologous substitutions can produce such strikingly different clinical phenotypes, we studied these mutations using the same approaches. Interestingly, the Carney mutants also demonstrated resistance to cAMP, but they expressed additional functional defects, including accelerated PRKAR1A protein degradation. These data demonstrate that cAMP binding defect is the common molecular mechanism for resistance of PKA activation in acrodysostosis, but the existence of several independent mechanisms leading to constitutive PKA activation in Carney complex.

Introduction

Many hormones and factors act on cells via specific G-protein coupled receptors to activate Gs proteins and adenylyl cyclases and result in the production of cAMP (1-3). The most studied target of cAMP is the cAMP-dependent protein kinase (PKA) (2). When in the inactive state, this serine/threonine kinase is composed of two catalytic subunits associated with two regulatory subunits. Binding of cAMP to the regulatory subunits results in the dissociation of catalytic subunits from the tetrameric complex and their activation (4). PRKAR1A is the most abundant and ubiquitous of the four known regulatory subunits (5). This 381 amino acid protein encompasses four identified domains, ordered as follows from the N-terminus: the dimerization domain, an "inhibitor sequence" that interacts with the catalytic subunit, the cAMP-binding domain A, and the cAMP-binding domain B (6).

Inactivating mutations of PRKAR1A have long been recognized as a genetic cause of Carney complex, a multiple endocrine neoplasia syndrome associating pigmented micronodular adrenal hyperplasia, cardiac myxoma, lentiginosis, pituitary tumors and other abnormalities (7, 8). Most of the mutations are nonsense or insertion/deletion mutations resulting in nonsense mediated decay (NMD) of the mRNA and absence of protein translation (9). The haploinsufficiency results in constitutive activation of PKA as the consequence of increased concentrations of free catalytic subunits. In a very limited number of cases, Carney complex is associated with a missense mutation in PRKAR1A, some occurring in the cAMP-binding domains. Studies evaluating the functional consequences of these missense mutations indicated that, beside haploinsufficiency, altered PRKAR1A function can elevate PKA activity (10).

Recently, we identified a unique mutation in the gene coding PRKAR1A in three unrelated patients with acrodysostosis, a heterogeneous group of rare skeletal dysplasia (11). The patients also presented resistance to several hormones, including PTH and TSH, and were defined as having acrodysostosis type 1. The mutation was a nonsense mutation of the last exon of PRKAR1A producing a truncated protein with a partial deletion of the cAMP-binding domain B. Functional characterization of this mutant protein demonstrated that it associated normally to the catalytic subunits, but an alteration of the dissociation of the subunits by cAMP was observed as the consequence of a reduced affinity of the PRKAR1A mutant to cAMP binding (11). Subsequently, other mutations of PRKAR1A have been identified in patients with acrodysostosis, including both missense and nonsense mutations, all of which were localized in one of the two cAMP-binding domains of the protein (12-16). All reported patients with PRKAR1A mutations presented hormonal resistance, by contrast to patients affected with acrodysostosis type 2 (caused by PDE4D mutations) (17). Assessment of the functional consequences of recently described PRKAR1A mutations has not been reported. Interestingly, two of these mutations involve amino acids (Ala213 and Gly289) for which alternative amino acid substitutions have been reported to cause the Carney complex phenotype (10). The reason why these alternative substitutions at the same site produce such strikingly different phenotypes has also not been previously explored.

To address this issue, we have evaluated the functional characteristics of PRKAR1A regulatory subunits carrying eight different mutations identified in patients with acrodysostosis and compared the results with those obtained for the two alternative mutations involved in the Carney complex.
Using a CRE-luciferase expression system, we have assessed the ability of cAMP to induce PKA-dependent protein expression. Using BRE techniques, we quantified the ability of regulatory subunits to bind to catalytic subunits in the absence of cAMP, and to dissociate from the catalytic subunits following exposure to cAMP analogs. For selected mutants we evaluated degradation rates using luciferase-PRKAR1A fusion proteins. Finally, we sought correlations between the functional defects observed and selected clinical characteristics of the patients.

**Materials and Methods**

**PRKAR1A mutations and patients**

Eight PRKAR1A mutations causing acrodysostosis with hormonal resistance and two causing the Carney complex were studied. Six acrodysostosis PRKAR1A mutations, all localized in cAMP-binding domain B, were previously identified and reported by us together with the patient's clinical, biochemical and skeletal phenotype (11, 13) (Gln285Arg, Gly289Glu, Ala328Val, Arg335Leu, the recurrent Arg368X mutation, and Gln372X ). The mutation Ala213Thr, localized in the cAMP-binding domain A, was reported by Muhn et al. (15). The mutation Tyr175Cys (c.524A>G), also localized in the cAMP-binding domain A, has not been previously reported. The affected patient was referred at the age of nine years for evaluation of brachydactyly. She presented with bilateral brachymetacarpal and metatarsal bones, affecting the IV and V metacarpal bones, and the II, III, IV and V metatarsal bones. Cone-shaped epiphyses and chunky bones were noted, but no widening of the lumbar interpediculate distance or ectopic ossification was observed. Her height was 1m30 (+1SD) and her weight was 30 kg. Biochemical and endocrine studies revealed increased serum PTH (106 pg/mL, normal range: 10-65); increased serum TSH (8.1 µIU/mL, normal: <5); normal T4 (14 pmol/L, normal range: 10-18); normocalcemia (2.33 mmol/L); slightly increased phosphatemia (1.6 mmol/L, normal range: 1-1.5 mmol/L); urinary cAMP at the upper limit of the normal range (0.95 micromol/mmol creatinine, normal range: 0.3-0.99). The parents showed neither brachydactyly or abnormal endocrine functions. Informed written consent was obtained from the patient’s parents. Analysis of the GNAS locus, including sequencing of GNAS exons 1 through 13 and evaluation of methylation at the four differentially methylated regions gave normal results (data not shown), ruling out abnormalities in the expression or function of the Gs α protein coded by this locus, which can produce similar developmental and skeletal features (pseudohypoparathyroidism type 1a). Exons and intron–exon junctions for the PRKAR1A gene (NM_002734) (12 exons) were amplified using intronic primers, and PCR products were analyzed by direct nucleotide sequence analysis as previously described (11). This mutation was not seen in the mother (the father's DNA was not available) or in 200 controls, and has not been reported in available databases (Single Nucleotide Polymorphism performance database/National Center for Biotechnology Information, 1000 genomes, exon variant servers, Human Genome Mutation Database, PRKAR1A mutation database). Correlations between clinical and molecular phenotype were performed using the clinical data reported in (11, 13) for the Gln285Arg, Gly289Glu, Ala328Val, Arg335Leu, Gln372X, and Arg368X mutations, described above for the Tyr175Cys mutation, and in Muhn, et al. (15) for the Ala213Thr mutation. The two homologous mutations that result in the Carney complex, Gly289Trp and Ala213Asp were reported by Greene et al. (10).

**Constructions and cell culture**

As previously described (11) the entire cDNA coding sequence of the human PRKAR1A and the mouse PRKACA genes were obtained by RT-PCR and subcloned in either pCDNA3.1 (Invitrogen) or pRLucN2(h) (Biosignal, PerkinElmer, Life Sciences) plasmids for PRKAR1A and pYFP-C3 (Clontech/BD Bioscience) for PRKACA. Site directed mutagenesis introducing the eight mutations associated with acrodysostosis and the two mutations associated with Carney complex was performed using the Quick Change site-directed mutagenesis kit (Stratagene) on both pCDNA-PRKAR1A and pR-PRKAR1A-Luc plasmids. The list of oligonucleotides used for PCR and site-directed mutagenesis are available upon request. All the constructions were verified by sequencing of the entire coding sequence and plasmid promoter. These plasmids were transfected alone or in combination in HEK293 cells (ATCC, USA).
using Effectene according to the manufacturer’s instructions (Qiagen). Two days later, the transfected cells were either lysed using Passive lysis buffer (Promega) and frozen or used to perform experiments described below.

**Western blotting**

Western blotting was performed using cell lysates obtained from HEK293 cells transfected with the empty plasmid or with the different PRKAR1A mutants subcloned in pCDNA3.1. Proteins were solubilized in MOSLB buffer (50mM NaPyrophosphate, 50mM NaF, 50mM NaCl, 5mM EDTA, 5mM EGTA, 100mM NaVO₄, 10mM Hepes pH7.4 and 0.1% Triton X100) with Complete mini protease inhibitor cocktail tablets (#11836153001, Roche). Ten to 40 micrograms of protein in Laemmli buffer were electrophoresed into a 10% acrylamide gel and then transferred onto a 0.2µm nitrocellulose membrane (GE Health Care BA83), incubated with anti-PKA[R1] monoclonal antibody (1/500; #610166 Transduction laboratory) or GAPDH polyclonal antibody (1/1000, #Ab37168, Abcam) in Tris Buffered Saline/Tween (137 mM NaCl, 20 mM Tris, 0.1% Tween 20, pH 7.6) + 5% nonfat milk. Secondary anti-mouse (1/5000) and anti-rabbit (1/10000) antibodies were incubated also in TBST + 5% nonfat milk and bands were revealed using an ECL kit (Super Signal, West Pico, chemiluminescence substrate, Thermoscientific, #34080).

**PKA-induced transcriptional activity**

Transcriptional PKA activity was measured as previously described (11). Briefly, HEK293 cells were co-transfected with 225 ng of a pCDNA3.1 construct encoding WT PRKAR1A or the various mutants; 250 ng of the pCRE-Luc plasmid (Stratagene), containing a firefly luciferase reporter gene under control of a CRE-responsive element; and 25 ng of the pRL-TK plasmid (Promega), containing a constitutively expressed *Renilla* luciferase reporter gene, which served as an internal control for transfection efficiency. Cells were either untreated (basal PKA-induced transcriptional activity) or treated with 0.3, 1, 5 and 10 µM forskolin (Sigma-Aldrich) in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich). Luciferase activity was determined 6 h after forskolin addition as described previously (11) using a Centro LB 960 luminometer (Berthold). For each culture, the ratio of firefly luciferase activity to *Renilla* luciferase activity was calculated to correct for transfection efficiency. Results were expressed as the fold-increase in CRE-luciferase activity relative to IBMX-treated cultures not stimulated with forskolin. To evaluate the EC₅₀ for forskolin stimulation, pooled data for each mutant relating the forskolin dose and the increase in CRE-luciferase activity was fitted to a sigmoid curve with variable slope.

**Interactions of PRKAR1A mutants with catalytic subunits using BRET technique**

The apparent affinity of wild type and the different mutant PRKAR1A subunits for the PRKACA subunit was evaluated by BRET. In each experiment, a fixed amount of PRKAR1A-luciferase plasmid (which varied from 1 to 5 ng in different experiments and for the different mutants) was transfected in HEK293 cells (6-well plates) in association or not with increasing amounts of the YFP-PRKACA plasmid (1 to 100 ng) as described (11, 18). For each transfection point, the luciferase, YFP and BRET signals were measured using a Mithras LB940 plate reader (Berthold technology). The results are expressed as the percentage of maximal BRET signal for a given ratio of YFP/Luciferase. The specific interaction between the regulatory and catalytic PKA subunits is identified by a hyperbolic increase of the BRET signal up to a plateau corresponding to the saturation of all donor molecules. BRET₅₀ corresponds to the binding of 50% of BRET donor molecules, and represents an indirect quantitative measurement of the affinity between the two subunits (19).

**Dissociation of PKA interactions by cAMP analogs using BRET technique**

The capacity of cAMP analogs to dissociate the wild type or mutant PRKAR1A-PRKACA complex was also tested using BRET. In each experiment, a fixed amount of PRKAR1A-luciferase plasmid (which varied from 20 to 30 ng in different experiments and for the different mutants) was used to transfect cells in a 60% confluent T-75 flask with or without a saturating amount of YFP-PRKACA plasmid (100 to 250 ng). The BRET signal in absence of YFP-PRKACA is considered 0% and 100%
in presence of YFP-PRKACA without cAMP analog. Increasing concentrations of cAMP analogs (10^{-10} M to 10^{-5} M) reduce the maximal BRET signal resulting in a sigmoid dose/response curve whose EC_{50} is proportional to the PRKAR1A affinity for this analog. The three tested analogs were: 8-Bromo-cAMP, known to bind with the same affinity to the A and B domains of PRKAR1A; 8-PIP-cAMP, binding preferentially to the A domain; 8-AHA-cAMP, binding preferentially to the B domain (20, 21).

**Degradation of PRKAR1A mutants**

Analysis of the PRKAR1A mutant degradation takes advantage of reliable and accurate measurement of the PRKAR1A-luciferase recombinant protein. Therefore, low amounts of this protein can be expressed and its degradation analyzed, avoiding the artifact of overexpression. The degradation rate of the untagged WT PRKAR1A evaluated by Western blotting and that of WT PRKAR1A-luciferase evaluated by luminescence gave concordant results (see Figure 8C). For each mutant, 10 to 30 ng of plasmid was used to transfect HEK293 cells in a 60% confluent T-75 flask. Twenty-four hours after transfection, the cells were trypsinized and seeded in 6-well plates. Twenty-four hours later, cycloheximide (20µg/mL) was added to half of the wells in order to block protein synthesis. Cells, treated or not with cycloheximide were lysed 0, 3, 6, 9 and 20h after the addition of cycloheximide and the luciferase signal was measured in presence of coelenterazine using a Mithras LB940 (Berthold technology). Each experiment was done in duplicate and repeated at least 3 times for each mutant. Degradation was calculated as the reduction of the luminescent signal in presence of cycloheximide compared to the luminescent signal at t=0.

**Three dimensional modeling of PRKAR1A mutants**

Human PRKAR1A was modelled as previously described (11). Molecular dynamic simulations using CHARMM29 force field were applied on human WT and mutant PRKAR1A 3D models in VEGAZZ (www.ddl.unimi.it/vegazz/) and NAMD software for 1 ns to check general reasonability of the modeled structure (22). The low frequency normal modes of human WT and mutant PRKAR1A 3D models were performed in elNémo software (http://www.sciences.univ-nantes.fr/elnemo/start.html). In order to establish the positioning of cAMP in the binding pocket of domain A or domain B of human PRKAR1A mutants, cAMP was docked into human WT PRKAR1A domain A or B according to the structural data from the X-ray structure of bovine PRKAR1A (23). The positioning of cAMP was then refined by calculation using Autodock 4.0 software (http://autodock.scripps.edu). Docked cAMP in domain A or B of WT and mutant PRKAR1A was submitted to energy minimization using MMFF94 force field. The rmsd (ligand) and binding pocket surface of the mutant PRKAR1As were estimated and compared to values obtained for WT PRKAR1A. Positioning of the side-chains of substituted residues in single mutants was determined using Chimera software (http://www.cgl.ucsf.edu/chimera/) and the Dunbrack rotamer library. Analysis and representation of ligand conformation in the domain A and B binding pockets of PRKAR1A was performed using Chimera software.

**Statistical analysis**

All results are expressed as mean ± SD unless otherwise indicated. For studies evaluating CRE-luciferase activity, the activity in cells expressing some PRKAR1A mutants was still increasing exponentially at 10 µM forskolin, the highest dose tested, and the EC_{50} could not be accurately determined. In these cases, the EC_{50} is reported as ">5 µM" and were evaluated using the Kruskal-Wallace test as implemented in R (version 3.1.2). Post-test comparisons, performed only if P < 0.05, were made using the nonparametric Wilcoxon matched-pairs test. Fold-stimulation was evaluated by analysis of variance. Post-test comparisons, performed only if P < 0.05; were made by pair wise t test. Data from BRET analysis was evaluated by analysis of variance. Post-test comparisons, performed only if P < 0.05 were made using the paired t-test with unequal variance. Clinical parameters in patient groups were compared using the Mann-Whitney test.

**Results**

The functional consequences of eight PRKAR1A mutations resulting in
acrodysostosis, comprising six novel missense mutations, one novel nonsense mutation (12-16) and the initially described Arg368X mutation (11) were characterized on the cAMP/PKA signaling pathway.

Expression of the PRKAR1A mutants
Following overexpression in HEK293 cells, all of PRKAR1A mutants were easily identifiable by western blotting (Figure 1). Most of the mutants had an apparent MW of 48kDa, comparable to the WT PRKAR1A protein and to the expected size of the protein. The two nonsense mutants (Arg368X and Gln372X) produced truncated proteins with a reduced MW. Interestingly, two of the missense mutants had either a slightly increased (Gly289Glu) or reduced (Gln285Arg) apparent MW, suggesting a change in the molecular structure that had been preserved during SDS-PAGE.

Acrodysostosis PRKAR1A mutants exhibit reduced PKA activity
We first analyzed the PKA-induced transcriptional activity in HEK293 cells overexpressing WT PRKAR1A or the eight mutant PKA subunits following forskolin stimulation using a CRE-luciferase reporter system. In the absence of forskolin, luciferase activity was low in transfected cells, and not significantly different from that of cells transfected with an empty vector (data not shown).

The dose-response curves for cells expressing the mutant PRKAR1A subunits were all shifted to the right compared to that observed for cells expressing the WT PRKAR1A subunit (Figure 2A). Following stimulation with low doses of forskolin (0.3 and 1 µM), the fold-increase in CRE-Luciferase activity was low in transfected cells, and not significantly different from that of cells transfected with an empty vector (data not shown).

Dissociation of PKA subunits induced by cAMP analogs
Since the major role of cAMP is to dissociate the regulatory subunits from catalytic subunits of PKA, thereby liberating the enzymatically active subunits, it was interesting to analyze the dose-dependent reduction of the BRET signal using cAMP analogs. To refine the analysis of cAMP action, three different cAMP analogs were studied: 8-PIP cAMP, 8-AHA cAMP and 8-Bromo cAMP, which interact more specifically with the PRKAR1A cAMP-binding domains A, B or both domains, respectively (20, 21).

Using the non-specific analog 8-Bromo cAMP, the dissociation of PRKAR1A subunits carrying mutations identified in acrodysostosis patients was significantly less efficient than that of WT PRKAR1A subunits (Figure 4 and Table II). The EC_{50}s for this analog were significantly increased for all of the mutant subunits. This result strongly suggests that the common molecular alteration of mutants in acrodysostosis is a reduction of cAMP binding, resulting in a reduced efficiency of cAMP-induced dissociation of the regulatory PKA subunits and activation of the catalytic PKA subunits.

Except for one mutant (Gly289Glu), the results obtained using the cAMP analogs specific for cAMP-binding domains A or B...
indicated that mutations in a given cAMP-binding domain specifically affected the binding of cAMP to that domain. Thus, using 8-AHA cAMP (B-domain analog), the PRKAR1A subunits carrying mutations in domain B, but not those with domain A mutations, showed significant (Gln372X, Arg368X, Arg335Leu, Ala328Val, Gly289Glu) or nearly significant (Gln285Arg) increases in EC\textsubscript{50} (Figure 4 and Table II). Conversely, using 8-PIP cAMP (A-domain analog) the two PRKAR1A subunits carrying mutations in domain A (Tyr175Cys and Ala213Thr) showed significant increases in EC\textsubscript{50} using this analog (Figure 4 and Table II). Only the Gly289Glu mutation in domain B showed also a significant increase of 8-PIP cAMP EC\textsubscript{50}.

Comparison of molecular phenotypes after substitutions of Ala213 and Gly289 causing either acrodysostosis or Carney complex

Among the few reported missense mutations in PRKAR1A causing the Carney complex, two (Ala213Asp and Gly289Trp) (10) affect amino acids for which alternative substitutions cause acrodysostosis (Ala213Thr and Gly289Glu). In an effort to understand how mutations of the same amino-acid cause such strikingly different phenotypes, we compared the two sets of mutations using the techniques described above.

PKA-induced transcriptional activity was reduced in cells overexpressing the Gly289Trp Carney mutant, as was observed for cells overexpressing the Gly289Glu acrodysostosis mutant (Figure 5). In contrast, the Carney-associated mutant Ala213Asp presented a specific pattern, with transcriptional activity comparable to the WT in cells treated with low dose of forskolin (0.3 \( \mu \)M), but a profound decrease in PKA-induced transcriptional activity following stimulation with higher doses of forskolin (Figure 5).

Using BRET, both Carney-associated mutants (Ala213Asp and Gly289Trp) demonstrated a hyperbolic and saturable association curves with corresponding BRET\textsubscript{50} values similar to those seen for WT PRKAR1A, and Ala213Thr and Gly289Glu mutants causing acrodysostosis (Figure 6 and Table I).

The dissociation of PKA subunits by cAMP analogs was also evaluated by BRET for the same four mutant regulatory subunits (Figure 7). For the Carney-associated mutant Gly289Trp the results show a significant impairment of subunit dissociation for all three cAMP analogs, similar to that observed for the acrodysostosis-associated mutant Gly289Glu, although the defect was somewhat less severe than for the Gly289Glu mutant. For the Carney-associated mutant Ala213Asp a major defect in subunit dissociation was observed in response to high doses of 8-PIP cAMP and 8-Bromo cAMP but not 8-AHA cAMP, consistent with its localization within the cAMP binding domain A. The impairment in subunit dissociation seen at high doses of 8-PIP cAMP and 8-Bromo cAMP was more severe than that observed for the acrodysostosis-associated mutant Ala213Thr, similar to the greater impairment of PKA activation for Ala213Asp mutant compared to Ala213Thr for high doses of forskolin. Surprisingly, in cells treated with low doses (0.1 to 1nM) of 8-Bromo cAMP, the dissociation of the Ala213Asp mutant seems to be greater than that of the WT PRKAR1A subunit. This observation raised the possibility that this mutant permits an increased, albeit low level of dissociation of PRKAR1A from PRKACA in the presence of low concentrations of cAMP, but a greater extent of dissociation at higher cAMP concentrations is strongly impaired due to the effect of the mutation on the cAMP binding domain A.

Since most of the Carney-associated mutations are nonsense mutations resulting in mRNA degradation by NMD (7, 8), we also investigated if the two present mutations can result in accelerated protein degradation. As shown in Figure 8 the Carney-associated mutant Ala213Asp presents a reduced degradation rate, similar to those of the two acrodysostosis-associated mutants Gly289Glu and Ala213Thr, as expected for regulatory subunits with impaired dissociation from catalytic subunit (2, 11, 26). In contrast, the Carney-associated mutant Gly289Trp presents a significantly accelerated rate of degradation, offering a likely explanation for its association with the Carney complex.

We also analyzed PRKAR1A structural consequences of Ala213 and Gly289 mutations by 3D-modeling. As indicated by analyses of trajectoires using 1ns molecular dynamics simulations (rmsd < 0.2 Å), substitution of Ala213 and Gly289 by aspartate or threonine residues and tryptophan or...
glutamic acid residues, respectively, do not directly impact the structure of the PRKAR1A subunit.

The glycyl residue at position 289 is a quite flexible amino-acid. A small increase of flexibility was observed for the Glu substitution causing acrodysostosis (rmsd_{wt} = 0.0087 vs rmsd_{G289E} = 0.0099), whereas a clear loss of flexibility was found for the Trp substitution causing Carney complex (rmsd_{wt} = 0.0087 vs rmsd_{G289W} = 0.0024), as calculated by the low frequency normal modes. These changes in flexibility may impact the cooperativity between cAMP A and B domains of the protein, since the Gly289 residue is located in the extended hydrophobic surface forming a contact between the 2 domains.

The Ala213 is located in the site of affinity labelling with 8-N3-cAMP (sequence 211-213) of the WT PRKAR1A cAMP-binding domain A (27). Interestingly, Ala213 is close to the Arg211 residue, which had a crucial role in the interaction network involved in cAMP binding in domain A (Figure 9, panel A). The substitution of the alanine residue by an aspartate (Ala213Asp) introduces two new negative charges in the binding site of domain A (Figure 9, panel B), resulting in the formation of two hydrogen bonds between Arg211 and Asp213 (Figure 9, panel B) and disrupting ionic bonds between: i) Asn173, Asp172 and Arg211; and ii) Ala213 and the cAMP molecule (27). The inability to dock the cAMP molecule into the molecular model containing this Ala213Asp mutation reinforces this hypothesis (data not shown). The acrodysostosis-associated Ala213Thr substitution also modified this interaction network (Figure 9, panel C), but modelling suggested that the affinity of this mutant for cAMP would be intermediate between that of WT PRKAR1A and the Ala213Asp mutant.

Clinical and molecular phenotype correlations in acrodysostosis

Although all PRKAR1A mutations occurring in acrodysostosis impaired its function, heterogeneity in the level of the functional defects was observed. Thus, we sought to identify correlations between the intensity of the hormonal resistance/clinical phenotype and the PKA molecular phenotype of the patients.

To do so, we divided the patients into two groups based on forskolin-induced Cre-luciferase activity: those for whom the fold-stimulation over basal was (Group A: Gln372X, Arg368X, Arg335Leu) or was not (Group B: Ala328Val, Gly289Glu, Gln285Arg, Ala213Thr, Tyr175Cys) significantly reduced compared to that of wild-type PRKAR1A following stimulation with both 5 and 10 µM forskolin. Hormonal resistance to PTH, but not TSH, was significantly greater in Group A, as indicated by a greater increase in serum PTH levels (Figure 10). No significant difference was observed between the two groups for serum calcium and phosphate (data not shown).

Discussion

In this study, we have characterized the functional consequences of eight different mutations in the gene coding PRKAR1A identified in patients with acrodysostosis. Our results indicate that in general terms, the overall mechanism responsible for acrodysostosis is similar in patients expressing different mutations. In each case, the mutation impaired the ability of cAMP to dissociate the regulatory and catalytic subunits of PKA, thereby reducing the intensity of PKA signaling induced after stimulation of cells with low to moderate amounts of cAMP. None of the mutations appeared to influence the binding of PRKAR1A to the catalytic subunits in the absence of cAMP, regardless of whether the mutations were missense mutations or nonsense mutations resulting in truncated regulatory subunits. In addition, mutations occurring in cAMP-binding domains A and B cause defects in PKA signaling of similar magnitude.

Despite these overall similarities, several differences were observed while comparing these variants providing new insights into the molecular pathogenesis of this clinical syndrome. Our results using BRET to evaluate the dissociation of regulatory subunits supported the idea that mutations occurring in the two cAMP-binding domains appeared to act locally, impairing cAMP-binding specifically to the involved domain. Mutations affecting domain A resulted in significant increases in the EC_{50} for 8-PIP cAMP, an analog that binds preferentially to domain A, but did not increase the EC_{50} for 8-AHA cAMP, which binds preferentially to domain B. All mutations affecting domain B resulted in significant increases in the EC_{50} for 8-AHA cAMP. Interestingly, however, the Gly289Glu mutation, but not the other mutations affecting
domain B, did increase the EC₅₀ for 8-PIP cAMP. It is conceivable that the Gly289Glu mutation induced conformational changes that could influence the structure of both cAMP-binding domains not detected by the present modeling studies. Indeed, the cAMP-binding domain B is the “gatekeeper” for PKA activation, as binding of cAMP to this domain is required to induce conformational changes necessary to expose the cAMP-binding domain A (27, 28). Thus, even though 8-PIP cAMP binds preferentially to domain A, sufficient binding to domain B is likely required to render the domain A accessible. Some mutations in domain B, such as Gly289Glu, may more strongly inhibit 8-PIP cAMP binding to domain B and/or impair the subsequent rearrangement required to expose domain A, resulting in an increase in the EC₅₀ for this analog. In this regard, the Gly289Glu mutation, located in a loop connecting the β₂ and β₃ strands and included in a hydrophobic region of domain B in contact with domain A, appears to alter the flexibility of the protein and induces changes which might alter the dynamic cooperativity of the two domains.

An important finding in our study was the observation that although all the PRKAR1A mutations reduced PKA signaling in response to cAMP, the extent of the reduction could be different depending on the mutations. These findings raise the possibility that the clinical characteristics observed in acrodysostosis may be, at least to some extent, mutation-dependent. Consistent with this idea, we found that patients carrying mutations that led to greater increases in the EC₅₀ for forskolin-induced CRE-Luciferase activity had, as a group, significantly higher plasma PTH levels. Further studies are needed to confirm this finding and evaluate the extent of the influence of functional differences in PRKAR1A activity on other clinical features of acrodysostosis, including skeletal dysplasia, growth, body weight and resistance to other hormones.

Carney complex is due to PRKAR1A mutations causing inappropriate constitutive activation of the catalytic subunits of PKA. In the large majority of the cases, nonsense mutations or insertions/deletions trigger NMD, resulting in protein haploinsufficiency of the regulatory subunit (7, 8). In the case of the two Carney missense mutations studied here, premature degradation of mRNA by NMD cannot be invoked. Possible alternative explanations for constitutive activation of PKA include: i) an abnormally high affinity of PRKAR1A for cAMP, ii) an impaired association of PRKAR1A and PRKACA under basal conditions, iii) inappropriate activation of PKA in response to low physiological concentrations of cAMP or iv) an accelerated degradation of the protein.

A previous report analyzing the molecular defects of Carney-associated missense mutations identified abnormal binding between the two PKA subunits and cAMP as the causes of increased PKA activity (10). Using different material and methods, we investigated two of the mutants (Ala213Asp and Gly289Trp) since the same positions are also mutated in acrodysostosis. This comparison provides new insights, indicating various mutation-dependent mechanisms for PKA activation.

The Carney-associated mutant Ala213Asp is located in the cAMP-binding domain A and, like the Ala213Thr mutant occurring in acrodysostosis, resulted in a markedly impaired binding of 8-PIP cAMP, but not 8-AHA cAMP, blunted PKA-induced transcriptional activity in response to high doses of forskolin and reduced protein degradation pattern. Several findings, however, supported the possibility that the Carney-associated Ala213Asp mutation, but not the acrodysostosis mutation Ala213Thr mutation presents a peculiar phenotype. The domain A of this mutant has a markedly impaired ability to bind cAMP, as suggested both by BRET experiments and 3D-modeling. This alteration does not change the basal interaction between the regulatory and catalytic subunits but, in addition to impairing cAMP binding to domain A, may also facilitate enzyme activation in the absence of subunit dissociation or subunit dissociation to some extent in the presence of cAMP concentrations insufficient to activate wild-type PKA (29). Consistent with this scenario, we observed: i) no significant increase in basal PKA-induced transcriptional activity, ii) preserved PKA-induced transcriptional activity in cells stimulated with low-dose, but not high-dose of forskolin, and iii) relative increased dissociation of regulatory and catalytic subunits measured by BRET in response to low-dose 8-bromo cAMP. In this regard, the similarities of our results for the Ala213Asp with those previously published by...
Greene et al. (10) are noteworthy. In their dose-response curves, PKA activity for lysates from cells expressing WT PRKAR1A plateaued at 0.1 µM cAMP, whereas for lysates from cells expressing Ala213Asp, activity continued to increase up to 5 µM cAMP, consistent with a higher EC$_{50}$ for the Ala213Asp mutants, as also observed by us using the Cre Luciferase system. Conversely, following stimulation with low-doses of cAMP (0.005-0.01 µM) the PKA activity of extracts from cells expressing the Ala213Asp mutant was consistently greater than that of cells expressing WT PRKAR1A, a finding compatible with inappropriate PKA activation occurring at low suprabasal levels of cAMP.

The Carney-associated Gly289Trp mutant exhibited a reduced affinity for cAMP without any change in the basal interaction between PRKAR1A and PRKACA, findings similar to what was observed with the acrodysostosis-associated Gly289Glu mutant. The accelerated degradation of this mutant is the most probable explanation for haploinsufficiency. The binding of PRKAR1A to the catalytic subunits is thought to prolong its half-life, and the Gly289Trp mutant was relatively resistant to cAMP-induced dissociation, suggesting that the accelerated degradation of the Gly289Trp mutant occurs at an early step in the process of synthesis, folding and/or transport. This early and premature degradation of the Carney-associated Gly289Trp mutant probably prevails on the other acrodysostosis-like defects, explaining the clinical phenotype.

Altogether, our results and those reported by Greene et al (10) indicate that Carney-associated mutations induce modest molecular alterations, which are difficult to visualize following overexpression in heterologous cells using the present available tools. Nevertheless, different mechanisms, including accelerated protein degradation and abnormal association of the two subunits in the presence of physiological concentrations of cAMP, appear to be involved. Further studies are required to directly demonstrate that the Ala213Asp mutant permits abnormally high PKA activity in the presence of suboptimal concentrations of cAMP, despite impairing cAMP binding to domain A.

In conclusion, the data presented here show for the first time and unambiguously that mutations of the PRKAR1A observed in acrodysostosis all result in a reduced affinity for cAMP by this regulatory PKA subunit. Quantitative and qualitative differences in this defect are discernible for different mutations, and these differences may have clinical implications that require further evaluation. Mutations occurring at the same position in PRKAR1A as seen in acrodysostosis, but resulting in the introduction of another amino acid can profoundly modify the observed phenotype, resulting in Carney complex.
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AUTHOR CONTRIBUTIONS

YR, CLS, WAK, CA, AC and EC performed the experiments. JB, AL, CS and EC participated to the design of the project and analyzed the data. AC did the modeling analysis. CS and EC wrote the manuscript. All authors analyzed the results and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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FIGURE LEGENDS

Figure 1. Expression of mutant PRKAR1A subunits analyzed by Western blotting. Cell lysates were prepared from HEK293 cells transfected with plasmids resulting in the expression of WT PRKAR1A or PRKAR1A carrying the indicated mutations (Western blot A = WT, Arg368X, Gly289Glu, Ala328Val, Arg335Leu, Gln372X and Gln285Arg; Western blot B = WT, Asp183Tyr, Ala213Asp and Gly289Trp; Western blot C = WT, Tyr175Cys and Ala213Thr). Soluble proteins were electrophoresed into 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and PRKAR1A (upper gel) and GAPDH (lower gel) were detected by chemiluminescence after sequential treatment of the membranes with specific primary antibodies, peroxidase-conjugated secondary antibodies and ECL substrate. Arrows show the migration of proteins of the indicated MW (kDa).

Figure 2. PRKAR1A mutations occurring in acrodysostosis impair PKA-induced transcriptional activity. Panel A: HEK293 cells were co-transfected with plasmids encoding i) WT PRKAR1A or the indicated mutants, ii) firefly luciferase reporter gene under control of a CRE-responsive element, and iii) a constitutively expressed Renilla luciferase reporter gene. Forty-eight hours later, cells were either left untreated or exposed to the indicated doses of forskolin in the presence of IBMX. Luciferase activity was determined 6 h after forskolin addition as described previously (11) and expressed as fold-stimulation over basal. To evaluate the EC_{50} for forskolin stimulation, pooled data for each mutant relating the forskolin dose and the increase in CRE-luciferase activity was fitted to a sigmoid curve with variable slope (Top panel). The median, range, and statistical analysis for data from the individual experiments are shown in the lower panel. †: paired results for four experiments are required to perform the Wilcoxon matched-pairs test; EC_{50} of these mutants was greater than EC_{50} of WT PRKAR1A in all experiments. Panel B: Histograms of the luciferase activity after stimulation with 0.3 and 1 µM forskolin. Results are presented as mean ± SEM for at least 3 independent experiments. Results were analyzed by ANOVA; post-tests (performed only if p<0.05) were made using the paired t-test with unequal variance. * p<0.05; **p<0.01. Values for 5 µM forskolin were not significantly different by ANOVA, and post-tests were not performed.

Figure 3: Donor saturation assay analyzed by BRET for the WT and the seven mutated PRKAR1A variants associated with acrodysostosis. The specific interaction between the catalytic and regulatory subunits is identified by a hyperbolic increase of the BRET signal expressed as a percentage of maximal BRET (Y) as a function of increases in the YFP/luciferase ratio (X).

Figure 4. Effect of PRKAR1A mutations occurring in acrodysostosis on PKA regulatory and catalytic subunit dissociation. HEK293 cells were co-transfected with a fixed amount of plasmid resulting in the expression of PRKAR1A-luciferase constructs carrying either the WT PRKAR1A sequence or the indicated mutations with or without a saturating amount of plasmid resulting in the expression of YFP-PRKACA. Forty-eight hours later, cells were lysed and cell lysates were exposed or not to the indicated doses of 8-bromo cAMP (top), 8-AHA cAMP (middle), or 8-PIP cAMP (bottom), and the BRET signal was measured as described in the Materials and Methods. The signal in absence of YFP-PRKACA was considered as 0% and in presence of YFP-PRKACA without the cAMP analog as 100%. To evaluate the EC_{50}, pooled data for each mutant relating the 8 cAMP analog concentration and the BRET signal was fitted to a sigmoid curve with variable slope. Values for variants with mutations in domain A are connected by dashed lines. The mean, SD, and statistical analysis for data from the individual experiments are shown in Table II.

Figure 5. Comparison of cAMP-induced PKA transcriptional activity in cells expressing missense mutations of the same PRKAR1A amino-acids causing either Carney complex or acrodysostosis. CRE-Luciferase activity in HEK293 cells overexpressing WT or mutant PRKAR1A was measured as described in Figure 2 legend, and is expressed as the fold-increase over basal observed following stimulation with the indicated doses of forskolin. Results (mean ± SEM) for mutations causing Carney complex (solid bars) and acrodysostosis (hatched bars) are shown for
mutations affecting Ala213 (dark grey) and Gly289 (light grey), and for WT PRKAR1A (solid white bars).

**Figure 6**: Donor saturation assay analyzed by BRET for the WT PRKAR1A and the variants carrying mutations at position Ala213 and Gly289 associated with acrodysostosis and Carney complex. The specific interaction between the catalytic and regulatory subunits is identified by a hyperbolic increase of the BRET expressed as a percentage of maximal BRET (Y) as a function of increases in the YFP/luciferase ratio (X).

**Figure 7**: Comparison of the ability of cAMP analogs to dissociate PRKACA subunits from PRKAR1A subunits expressing missense mutations at the same amino-acids causing either Carney complex or acrodysostosis. The dissociation of catalytic and regulatory subunits of PKA induced by the indicated doses of 8-bromo cAMP (top panel), 8-AHA cAMP (middle panel) and 8-PIP cAMP (bottom panel) was evaluated by BRET as described in Figure 3 legend. Results are shown as the mean ± SEM for WT PRKAR1A (solid red lines) and PRKAR1A carrying mutations causing Carney complex (solid lines) and acrodysostosis (dotted lines) for the indicated mutations affecting Ala213 (purple lines) and Gly289 (blue lines), and for WT PRKAR1A (solid red lines).

**Figure 8**: Effect of missense mutations in the same amino-acids of PRKAR1A causing either Carney complex or acrodysostosis on the degradation of PRKAR1A. HEK293 cells were transfected with PRKAR1A-luciferase constructs, plated in duplicate wells, and 24h later cycloheximide was added to half of the wells. At the indicated times after addition of cycloheximide, luciferase activity was measured by luminometry. Results, expressed as the percentage of the time point=0 fluorescence, are the mean ± SEM for at least three independent experiments, each performed in duplicate. Shown are findings for WT PRKAR1A (solid black lines) and PRKAR1A carrying mutations causing Carney complex (solid lines) and acrodysostosis (dotted lines) for the indicated mutations affecting Ala213 (panel A) and Gly289 (panel B). Panel C: Comparison of WT PRKAR1A degradation using Western blot or luminometry. HEK293 cells were transfected with WT PRKAR1A or PRKAR1A-luciferase constructs, plated in duplicate wells, and 24h later cycloheximide was added. At the indicated times after addition of cycloheximide, the PRKAR1A protein was analyzed by Western blot (blue curve) or luciferase activity measured by luminometry (red curve). Results, expressed as the percentage of results at time point=0, are the mean ± SEM for at three to five independent experiments. (Unpaired Student t test * p<0.05, ** p<0.01, *** p<0.001).

**Figure 9**: Stick representations of models showing cAMP docking into the domain A binding pocket of PRKAR1A. Shown are models of the cAMP binding domain A for WT PRKAR1A (panel A), mutant Ala213Asp (panel B) and mutant Ala213Thr (panel C). Dashed lines represent ionic interactions. All images were generated with Chimera software (www.cgl.ucsf.edu/chimera/).

**Figure 10**: Hormonal resistance to PTH and TSH, in patients with acrodysostosis as a function of the impact of the causative mutation on cAMP-induced PKA transcriptional activity. Acrdysostosis patients were divided into two groups based on forskolin-induced Cre-luciferase activity: those for whom the fold-stimulation over basal was (Group A: Gln372X, Arg368X, Arg335Leu) or was not (Group B: Ala328Val, Gly289Glu, Gln285Arg, Ala213Thr, Tyr175Cys) significantly reduced compared to that of wild-type at both 5 and 10 µM forskolin. Plasma PTH (panel A) and TSH (panel B) are expressed as the fold-increase over the upper limit of normal for the assay used. Groups were compared using the Mann-Whitney test.
Table I: Apparent affinity of wild type and the different mutant PRKAR1A subunits for the PRKACA subunit.

|       | BRET50 (mean±SEM) | n  | P     |
|-------|-------------------|----|-------|
| WT    | 0.48 ± 0.08       | 5  |       |
| Tyr175Cys | 0.40 ± 0.15     | 3  | 0.65  |
| Ala213Thr | 0.22 ± 0.11     | 3  | 0.11  |
| Ala213Asp | 0.40 ± 0.15     | 4  | 0.65  |
| Gln285Arg | 0.48 ± 0.35     | 2  | 0.99  |
| Gly289Glu | 0.34 ± 0.17     | 4  | 0.45  |
| Gly289Trp | 0.51 ± 0.27     | 3  | 0.91  |
| Ala328Val | 0.28 ± 0.18     | 2  | 0.30  |
| Arg335Leu | 0.53 ± 0.43     | 2  | 0.85  |
| Arg368X  | 0.35 ± 0.14     | 5  | 0.46  |
| Gln372X  | 0.76 ± 0.19     | 2  | 0.17  |

n = number of experiments. P values are calculated by comparing the results of each mutant with the results for the wild type protein (unpaired Student’s t test)
### Table II. Dissociation of PKA regulatory and catalytic subunits induced by cAMP analogs

| MUTANT      | 8-bromo cAMP | 8-AHA cAMP | 8-PIP cAMP |
|-------------|--------------|------------|------------|
|             | EC₅₀ (nM)    |            |            |
|             | mean ± SD    | n P vs WT  | mean ± SD  | n P vs WT  | mean ± SD  | n P vs WT |
| WT          | 9.0 ± 4.1    | 9 -        | 1.0 ± 0.4  | 7 -        | 300.3 ± 135.4 | 8 - |
| Gln372X     | 21.2 ± 5.9   | 5 0.010    | 8.5 ± 3.2  | 5 0.010    | 176.8 ± 36.4  | 5 0.050 |
| Arg368X     | 20.9 ± 5.5   | 4 0.023    | 12.1 ± 5.6 | 4 0.044    | 553.5 ± 182.3 | 3 0.177 |
| Arg335Leu   | 21.1 ± 6.1   | 5 0.012    | 64.2 ± 21.9| 4 0.015    | 732.2 ± 1242.0| 5 0.526 |
| Ala328Val   | 23.6 ± 7.8   | 5 0.016    | 1.8 ± 0.4  | 4 0.017    | 374.5 ± 149.7 | 4 0.492 |
| Gly289Glu   | 182.2 ± 41.3 | 6 <0.001   | 67.7 ± 17.8| 4 0.007    | 3415.1 ± 2091.4| 7 0.011 |
| Gln285Arg   | 21.3 ± 5.7   | 5 0.008    | 2.4 ± 1.0  | 5 0.055    | 191.2 ± 80.6  | 5 0.122 |
| Ala213Thr   | 31.5 ± 13.1  | 6 0.011    | 3.3 ± 2.5  | 5 0.136    | 1948.0 ± 387.7| 4 0.004 |
| Tyr175Cys   | 35.3 ± 11.7  | 6 0.003    | 2.8 ± 2.3  | 4 0.273    | 1303.8 ± 238.8| 5 <0.001 |
Figure 2
Figure 3
Figure 5

Cre Luciferase Activity (fold-increase over basal)

0.3 μM forskolin

1 μM forskolin

5 μM forskolin

10 μM forskolin

WT
ALa212Asp
ALa213Thr
Gly289Trp
Gly299Glu

WT
CARNEY
ACRO

**
**
**

*
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10

**A**
PTH

- Fold increase over upper limit of normal
- Group A and Group B
- P < 0.01

**B**
TSH

- Fold increase over upper limit of normal
- Group A and Group B
- P = 0.09
Functional Characterization of PRKAR1A Mutations Reveals a Unique Molecular Mechanism Causing Acrodysostosis but Multiple Mechanisms Causing Carney Complex

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