Regulation of cAMP-dependent Protein Kinases

THE HUMAN PROTEIN KINASE X (PrKX) REVEALS THE ROLE OF THE CATALYTIC SUBUNIT αH-αl LOOP

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cAMP-dependent protein kinases are reversibly complexed with any of the four isoforms of regulatory (R) subunits, which contain either a substrate or a pseudosubstrate autoinhibitory domain. The human protein kinase X (PrKX) is an exemption with any of the four isoforms of regulatory (R) subunits, which is inhibited only by pseudosubstrate inhibitors, i.e. RIIα or RIIβ but not by substrate inhibitors RIIα or RIIβ. Detailed examination of the capacity of five PrKX-like kinases ranging from human to protozoa (Trypanosoma brucei) to form holoenzymes with human R subunits in living cells shows that this preference for pseudosubstrate inhibitors is evolutionarily conserved. To elucidate the molecular basis of this inhibitory pattern, we applied bioluminescence resonance energy transfer and surface plasmon resonance in combination with site-directed mutagenesis. We observed that the conserved αH-αl loop residue Arg-283 in PrKX is crucial for its R over RII preference, as a R283L mutant was able to form a holoenzyme complex with wild type RII subunits. Changing the corresponding αH-αl loop residue in PKA Ca (L277R), significantly destabilized holoenzyme complexes in vitro, as cAMP-mediated holoenzyme activation was facilitated by a factor of 2–4, and lead to a decreased affinity of the mutant C subunit for R subunits, significantly affecting RII containing holoenzymes.

The protein kinase A (PKA) holoenzyme is a heterotetramer composed of two catalytic (C)5 subunits kept inactive by a dimer of R subunits. Each R subunit monomer contains two tandem cAMP-binding domains, in which the sequential binding of two cAMP molecules releases an active C subunit (1). Two main classes of PKA isozymes, type I and type II, distinguishable by their R subunits, have been described. Crystal structures and solution scattering data provide evidence of a complex interaction network between C and R subunits as well as differences in global structure of PKA type I and type II holoenzymes (2–5).

Homo sapiens express the PKA R subunit isoforms RIIα, RIIβ, RIIα, and RIIβ and C subunits Ca, CB, and Cy. Another cAMP-dependent protein kinase is PrKX and possibly protein kinase Y (PrKY). PrKY is 94% homologous to PrKX, but shortened by 81 amino acids at the C terminus. On the genome level, PKRXX and PRKY are implicated in sex-reversal disorders (6). PrKX is being discussed as a phylogenetically and functionally separate enzyme (7, 8). In contrast to the ubiquitously expressed Ca subunit, PrKX is mainly active during embryonic organ development and cellular differentiation in hematopoietic lineages. It was found to be crucial for macrophage and granulocyte maturation (9, 10). PrKX was shown to be involved in renal development, regulating epithelial cell migration, ureteric bud branching, and induction of glomeruli formation (8, 11–13).

Ca and human PrKX differ by their selective holoenzyme formation in living cells, as PrKX is inhibited only by RIIα, but not by RIIα (14, 15). Here, we have tested all four human R subunits for the first time side by side and show that this so far unique property of R over RII preference with respect to autoinhibition appears to be an evolutionarily conserved feature of PrKX and at least four of its orthologs (Mus musculus Pkare, Drosophila melanogaster DC2, Trypanosoma brucei PKAC3, human PrKY), and possibly also Caenorhabditis elegans F47F2.1b.

Previously, we identified the R subunit autoinhibitory site as a main determinant for isoform-specific regulation of PrKA (15). We were able to gain significant binding of PrKX with RIIα solely by mutation of Ser-99 to Ala in the RIIα autoinhibitory domain (D9-site). Conversely, introducing an autophosphorylation site Ser or an Asp in the inhibitory domain of RIIα completely abolished binding to PrKX, but not to Ca. This led us to believe that at least one peripheral interaction interface could be non-functional in PrKX holoenzymes when compared with the Ca holoenzymes. We therefore set out to pinpoint this position in PrKX, applying mainly BRET-based cell interaction...
assays and SPR analyses with wild type and mutant C subunits. Our investigations led to the identification of residue Arg-283, located in the PrKX αH-αl loop, as important for the differential regulation of the PrKX subfamily, i.e. its selective autoinhibition by R1 subunits in living cells. Supported by biochemical and modeling evidence we provide evidence that an Arg at position 283 in PrKX or at position 277 in the mutant Cα interferes with holoenzyme stability and allosteric regulation of the kinases by disturbing a crucial interaction platform composed of activation loop (C subunit) and αA-helix (R subunit) residues.

**EXPERIMENTAL PROCEDURES**

**Protein Expression Vectors**—The oligonucleotides and vectors used for cloning of prakroyotic and eukaryotic expression vectors and for subsequent site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene) are provided under supplemental Table S2. Cloning of human PRKAR1A, PRKAR2A, PRKAC, and PRKX genes into the BRET vectors was published previously (15, 16). Mutagenesis of PrKX was performed using plasmid pFastBac HTb-PRKX (15) as the template. For protein purification, human Cα was subcloned into pHis5BA via pRSETg-hCα (supplemental Table S2). pKare was amplified from pCMVSPORT6-Pkare (RZPD, Berlin, Germany). PRKY was amplified from cDNA clone pCMV6-XL5-PRKY (accession number NM_002736.2) and subcloned in Rluc(h)-N2 and pRSETB, was amplified from a cDNA clone (OriGene, accession number VOLUME 285 • NUMBER 46)

**Protein Expression and Purification**—Expression and affinity purification of R subunits was carried out as described (18, 19). His6-tagged human Cα (His6-Cα) and His6-CαL277R were expressed overnight at room temperature in Escherichia coli BL21(DE3) (Novagen) and purified using a Talon affinity resin and standard conditions (Clontech). Expression and purification of His6-PrKX and His6-PrKX_L277R was performed as described (15).

**Spectrophotometric Kinase Activity Assay**—The specific activity of the recombinant PKA Cα was tested by the continuous enzyme-linked spectrophotometric method described by Cook et al. (20) using 260 μM of the synthetic substrate Kemptide (LRRASLG; Biosynth). 1 Unit/mg is defined as 1 μmol × min^-1 × mg^-1. Apparent activation constants (K_a) were determined with 10 or 20 nm reconstituted holoenzyme by adding varying concentrations of cAMP in assay buffer containing 1 mM ATP and 10 mM MgCl_2 (15).

**Surface Plasmon Resonance**—Methods for interaction analyses of Cα and PrKX with R subunits were published previously (15). Briefly, 200–300 resonance units (RU) of His6-Cα or His6-CαL277R in complex with the bovine (b) RII were covalently coupled on a modified Ni^{2+}-nitriilotriacetic acid (NTA) chip via primary amines. A dilution series (0.5 to 256 nM R subunit) was injected at a flow rate of 30 μl/min, and association and dissociation were recorded for 5 min each. A blank NTA surface was used as the control surface. Additionally, an injection of buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% Tween 20, 50 μM EDTA) was subtracted from each sensorgram. Surface regeneration was achieved by injecting 100 μM cAMP and 2.5 mM EDTA, diluted in running buffer. The rate constants for association (k_a) and dissociation (k_d) were fitted assuming a 1:1 Langmuir binding model using Biaevaluation 4.1 (Biacore AB). Equilibrium binding constants (K_d) were calculated by dividing k_d with k_a.

**Molecular Dynamics (MD) Simulations**—The structural models of Cα and CαL277R in complex with the bovine (b) RIIα were based on the available x-ray structures solved at 2.5 Å of resolution (Protein Data Bank code 2QVS (4)). The L277R mutant side chain was modeled to maintain the orientation of the wild type side chain in the experimental structures. Missing atoms were added assuming standard bond lengths and angles. The models were immersed in parallel piped water boxes, whose edges were ~9, 10, and 12 nm; sodium or chlorine ions...
Intracellular PKA霍洛酶调节

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Previous in vitro and BRET-based interaction analyses in living cells using Rα and RⅡa subunits identified human PrKX as a type Iα-specific PKA-like kinase (14, 15). To study binding patterns of PrKX-like kinases, we investigated binding of all four human R isoforms to PrKX, human PrKy, murine Pkare (31, 32), D. melanogaster DC2 (33), and a putative PrKX homolog from the protozoan parasite T. brucei (PKAC3, GenBank accession number AF253418 (34)). They were all cloned as N-terminal GFP2 fusions for eukaryotic protein expression. In analogy to the previously published protein expression constructs for human Rα and RⅡa (16), Renilla luciferase (Rluc) was genetically fused to the C terminus of RⅡβ and RⅡβ.

For BRET analyses in COS-7 cells, the five investigated PrKX-type kinase fusion constructs were co-transfected with each of the four human R subunits at a 1:1 ratio of plasmid DNA. Employing this BRET assay, a signal above the background indicates PKA holoenzyme formation, which can be reduced by increasing intracellular cAMP ([cAMP]). Furthermore, the BRET signal of a given interaction pair provides a quantitative measure of the amount of holoenzyme present in resting versus stimulated cell populations (17). Fig. 1 shows the protein-protein interaction analyses, where PrKX (A), PrKy (B), Pkare (C), a deletion variant of DC2 (ΔDC2 (D)) and PKAC3 (E) were each probed by BRET for binding to the four human R subunits. With DC2 we achieved BRET signals and PKAC3 (E) were each probed by BRET for binding to the four human R subunits at a 1:1 ratio of plasmid DNA.

RESULTS

Inhibition of PrKX and Orthologs by Human R Subunits—Previous in vitro and BRET-based interaction analyses in living cells using Rα and RⅡa subunits identified human PrKX as a type Iα-specific PKA-like kinase (14, 15). To study binding patterns of PrKX-like kinases, we investigated binding of all four human R isoforms to PrKX, human PrKy, murine Pkare (31, 32), D. melanogaster DC2 (33), and a putative PrKX homolog from the protozoan parasite T. brucei (PKAC3, GenBank accession number AF253418 (34)). They were all cloned as N-terminal GFP2 fusions for eukaryotic protein expression. In analogy to the previously published protein expression constructs for human Rα and RⅡa (16), Renilla luciferase (Rluc) was genetically fused to the C terminus of RⅡβ and RⅡβ.

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In this study, we focused on a P0-site mutant R subunit. The specific activity, intracellular and extracellular stabilization of Cα (E208A), all shaded in black. Non-conserved and/or experimentally addressed residues in this study are shaded gray and written in black (L277S), red (R283PrKX, R283Rluc, R284Arg), and yellow (P254PKAC3, E253PKAC3), B, BRET interaction analyses of wild type and PrKXR283L with wild type or RIIα-Rluc constructs. We went on to replace Arg-283PrKX to Leu and monitored holoenzyme formation using the BRET assay. This mutation had no effect on C subunit binding to any of the four human R subunits (data not shown), in agreement with a previous study investigating a Lys-285 to Pro mutant (39), suggesting a backbone rather than a side chain interaction between Lys-285 and Leu-366.

We then focused on an αH-αl loop residue that differs between PrKX and Cα, Arg-283PrKX and Leu-277Cα. Arg-283PrKX is conserved in all but one of the PrKX-like kinases investigated in this study and the corresponding residue Leu-277Cα is common to Cα and -orthologs (Fig. 2A and supplemental Fig. S1, B and C). We went on to replace Arg-283PrKX by Leu in PrKX and investigated the mutant protein for specific activity, intracellular and in vitro binding to wild type and P0-site mutant R subunits. The activities of the purified PrKX proteins were tested with a coupled spectrophotometric assay using the synthetic substrate Kemp tide (LRRASLG) (20). Results were almost identical for PrKX (14) and mutant PrKXR283L (1.4 ± 0.2 units/mg, data not shown).

As depicted in Fig. 2, D and E, changing Arg-283PrKX to Leu had a significant stabilizing effect on wild type RIIα and RIIβ subunit binding in living cells. SPR analyses using the corresponding purified proteins lead to an about 10–12-fold increase in binding affinity of PrKX to RIIα subunits (Table 1 and Fig. 3, C and D). A combined expression of PrKXR283L and P0-site mutants RIIα(S99A) or RIIβ(B114A) lead to BRET signals comparable with RIIα and RIIβ wild type holoenzymes (Fig. 2).

Table S1. From x-ray structural analysis of either RIα or RIIα in complex with the Cα subunit, the αH-αl loop region of the C subunit was proposed to be important particularly for binding of the RIIα subunit (3, 4). Residues Asp-276Cα and Thr-278Cα as well as the residues Thr-278Cα and Lys-285Cα were previously found to interact with the cAMP binding domain A of bovine RIα (Arg-352, Arg-355) and bovine RIIα (Arg-365, Leu-266) subunits (Fig. 2A). These αH-αl loop residues are conserved between PKA and PrKX-like kinases. To elucidate residues necessary for the interaction with RII subunits, we first mutated Lys-285 to Ala in Cα and monitored holoenzyme formation using the BRET assay. This mutation had no effect on C subunit binding to any of the four human R subunits (data not shown), in agreement with a previous study investigating a Lys-285 to Pro mutant (39), suggesting a backbone rather than a side chain interaction between Lys-285 and Leu-366.

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Intracellular PKA Holoenzyme Regulation

Table 1

Rate and equilibrium binding constants of PrkX/R and Ca/R subunit interaction derived from SPR analyses

|          | R1α  | R1β  | R1α  | R1β  | R1α  | R1β  | R1α  | R1β  |
|----------|------|------|------|------|------|------|------|------|
|          | kₐ (M⁻¹ s⁻¹) | kₐ (s⁻¹) | kₐ (s⁻¹) | kₐ (s⁻¹) | kₐ (s⁻¹) | kₐ (s⁻¹) | kₐ (s⁻¹) | kₐ (s⁻¹) |
| PrkX     | 2.6 x 10⁵ | 6.9 x 10⁵ | 2.2 x 10⁻⁵ | 2.8 x 10⁻³ | 0.8 | 4 | ~3–4 μM | ~5–10 μM |
| PrkXR283L | 5.9 x 10⁵ | 7.9 x 10⁵ | 2 x 10⁻² | 3.2 x 10⁴ | 3.2 x 10⁴ | 1.4 x 10⁻⁵ | 5.1 x 10⁴ |
| CaL277R  | 8.1 x 10⁵ | 2.2 x 10⁵ | 3.1 x 10⁻⁵ | 3.9 x 10⁵ | 0.1 | 0.17 | 0.77 | 0.79 |
| CaL277R  | 9.1 x 10⁵ | 2.4 x 10⁵ | 5 x 10⁻⁵ | 3 x 10⁵ | 0.2 | 0.33 | 5.2 | 3.5 |

Using SPR, a Kₐ value of 251 nm compared with ~3–4 μM (Table 1) translates into a ~12–16-fold increased affinity of PrkX/R283L and RI1αL277R. As depicted in Fig. 2, B and C, results of BRET assays employing either R1α or R1β in combination with wild type PrkX or PrkX/R283L display a similar holoenzyme dynamics. As previously shown for R1α wild type holoenzymes with Ca or PrkX (15, 16), wild type R1β holoenzymes with PrkX and PrkX/R283L do not respond with complete dissociation upon cAMP elevation in living cells (Fig. 2, B and C). In vitro, the affinity of R1β and PrkX/R283L measured by SPR increased by about a factor 3 (Table 1 and Fig. 3B), due to a reduction in the off-rate. The interaction of wild type PrkX with mutant R1α and R1β resulted in comparably high BRET values (Fig. 2, B and C and Ref. 15). Introducing a Ser residue at the P⁰-site in both type I R subunits prohibited binding of PrkX. In the case of R1α, but not R1β, the negative effect of this mutation was partially compensated by changing Arg-283PrkX to Leu (Fig. 2, B and C). This was also reflected by a 2-fold stronger in vitro binding of RI1αL277R to PrkX/R283L compared with PrkX (Table 1). In a previous study, we showed that interaction of PrkX with an in vitro phosphorylated RI1αL277R protein was completely abolished (15). From this we can conclude that Arg-283PrkX located in the α-h-α loop region prohibits high-affinity binding to and thus functional inhibition of PrkX by wild type R1I or mutant RI subunits in living cells.

Initial investigation of a putative ortholog of PrkX from C. elegans (F47F2.1b) did not lead to significant interaction with human R subunits in the BRET assay. We therefore reasoned that replacing the Arg at position 324 for Leu in F47F2.1b might stabilize the interaction with R subunits in analogy to human PrkX. This was indeed the case, as we gained interaction of the mutant C subunit with R1α and R1β subunits (supplemental Fig. S2A). The PKAC3 protein from T. brucei is the only putative PrkX ortholog investigated in this study that does not carry an Arg at the position corresponding to Arg-283PrkX but a Pro (Fig. 2A). We therefore investigated the interaction of the protein to R subunits after replacing Pro-254PKAC3 with Leu. Interestingly, interactions of wild type PKAC3 with either R1α or R1β subunits were reduced to background levels upon mutation of the C subunit, indicating the importance of the Pro residue for holoenzyme formation with this T. brucei C subunit (supplemental Fig. S2B).

Mutation of Leu-277Ca Influences Holoenzyme Activation—To test the hypothesis of a central role of Leu277Ca, which corresponds to Arg-283PrkX, in maintaining a stable PKA holoenzyme complex, we investigated the consequence of introducing an Arg at position 277 in Ca in vitro and in living cells. This mutation had no effect on the specific activity of the purified kinase, determined by the coupled spectrophotometric assay (20) (20 ± 1.1 units/mg, data not shown). We then tested the activation of reconstituted holoenzymes containing wild type and CaL277R with four R isoforms in vitro (Table 2). Intriguingly, the apparent activation constants (Kₐ) of all mutant holoenzymes tested were significantly reduced, in other words, the activation threshold for cAMP is lowered in the mutant holoenzyme complexes under in vitro conditions. The effects ranged from a reduction by factor 2 in the case of RI subunits up to factor 4 in the R1β containing holoenzyme. These results were obtained with proteins from two independent protein preparations.

Interaction analyses by SPR revealed that the affinity of the CaL277R subunit toward R subunits is reduced (Table 1 and Fig. 3, E–H). The effect is again more evident with RI1α subunits (factor 6.75 for RI1α, factor 4.4 for RI1β), compared with a factor 2 for R1α and R1β. The increased Kₐ values were due to faster off-rates with the CaL277R compared with wild type Ca (Table 1 and Fig. 3, E–H). We then went on to test the interaction of PKA Ca as well as CaL277R with wild type and mutant human R subunits in living cells using the BRET assay. In accordance to a previous study comparing the PKA-type Iα and PKA-type IIα holoenzyme dissociations upon cAMP elevation, we observed that R1α or R1β containing holoenzymes are less sensitive to activation by cAMP (Ref. 15, and data not shown), in contrast to the RI1α and R1β containing holoenzymes (Fig. 4, A and B), which readily activate. This almost dominant inhibitory effect of the RI subunits is lost by simple mutation of the autoinhibitory site from a pseudosubstrate to a substrate site (Ref. 15, and data not shown). In contrast, replacing the R1α or R1β subunit...
P₀-site Ser with Ala prohibits a complete activation by cAMP in the context of the living cell (Fig. 4, A and B). Interestingly, this inhibitory effect of the P₀-site mutation was lost, and [cAMP]ᵢ was again able to activate the holoenzymes significantly when the RIIα/β subunits were combined with the mutant C₇₂₇₇R (Fig. 5). In the case of the PKA type I holoenzymes, mutation of the C subunit αH–αl loop had no measurable effect on holoenzyme formation or dissociation in living cells (data not shown).

In summary, the mutation of Leu-277 in Cα leads to destabilization of the PKA holoenzyme in vitro, due to a reduced subunit affinity and activation at lower cAMP concentrations. This effect is stronger when investigating holoenzyme formation with RII subunits, thus resembling the situation in PrKX holoenzymes, which exhibit a strongly reduced binding affinity in vitro leading to no significant RII subunit binding in vivo. Under steady-state conditions in living cells, mutation of the C subunit at position Leu-277 to Arg has more subtle effects, influencing the stability of RII containing holoenzymes.

Modeling of the Cα Leu-277 to Arg Mutation—To gain insight into putative structural and regulatory consequences of changing Cα Leu-277 to Arg on the interaction network of PKA type II, we modeled the wild type and mutant complexes on the basis of the available experimental structure of the PKA RIIα/C complex (PDB entry code 2QVS (4)). The model was refined by means of MD simulations: 14 ns of MD were performed and root mean square deviations fluctuated at about 3 Å. In the wild type structure, Leu-277Cα is located in the intersubunit cleft, packed against the side chain of Arg-280Cα, which forms a salt bridge interaction with Glu-208Cα (40). On the other side of Leu-277Cα, the so-called intersubunit interface site 3 (3, 4) includes a salt bridge network of the activation loop and αH–αl residues formed by Arg-194/Asp-271bRII and Arg-245bRII (Fig. 5). This region is crucial for stabilizing the holoenzyme conformation as well as for the allosteric coupling in the PKA holoenzyme. For instance, the Arg-245/Asp-271bRII salt bridge plays a key role in coupling the cAMP binding domains

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**FIGURE 3.** Representative SPR sensorgrams of R subunit binding to immobilized His₆-tagged PrKX, PrKXR283L, Ca, and CaL277R. All C subunits were covalently coupled on Ni²⁺-NTA sensor surfaces. Shown are normalized binding curves, where 128 nM of each RII (A and E), RIIβ (B and F), RIIα (C and G), and RIIβ subunit (D and H) were injected at a flow rate of 30 µl/min in running buffer containing ATP/ Mg²⁺, except for PrKX, where an injection of 512 nM of the RIIα/RIIβ subunits is depicted (C and D). Insets in panels C and D represent original SPR data for the PrKX/RIIα and PrKX/RIIβ interactions.
of the R subunit (3, 41). In the complex, the tip of the activation loop of Cα, namely residues Lys-192-Gly-193-Arg-194, is packed in the intersubunit interface to form hydrophobic contacts with Ser-264 and Met-267 in bRIIα (Fig. 5A). These interactions are observed in the experimental structures and are reproduced in our simulations of the wild type complex. On the contrary, mutating Leu-277Cα to Arg affects the conformation of the Asp-271bRII side chains, which rotate toward and interact with Arg-277Cα (Fig. 5B). The interaction network of the site is affected so that the solvent exposure of the residues in the tip of the activation loop increases by about 45 Å² in the mutant complex, with respect to the wild type, indicating higher solvation of the intersubunit interface. Although the K_D values of the wild type versus mutant type IIα holoenzyme decreased only by a factor of 6.75, still being in the nanomolar range of affinity (Table 1), the theoretical structural analysis supports our experimental data, which showed a decrease in binding affinity of RII subunits to CαL277R and a facilitated activation of the mutant PKA holoenzymes.

**DISCUSSION**

Phylogenetic analyses performed on the kinase core (8) and the overall sequence of PrKX (supplemental Fig. S1, A and B, and Table S1) indicate that the kinase and its orthologs belong to the family of AGC kinases but are distinct from conventional PKA. Although the kinase core of PrKX shares significant overall homology to PKA Cα (~57% identity (8)), other regions, including the N and C termini (~23 and ~40% identity, respectively) have diverged. The N terminus is the least conserved part, even among PrKX-like kinases. Large insertions, for example, in DdpK2 from Dictyostelium discoideum (42, 43), and in DC2 from D. melanogaster (33) are characteristic for some PrKX-like kinases (supplemental Fig. S1A). This diversity is reflected in the phylogenetic analysis of the overall sequence (supplemental Fig. S1A), the kinase core, and the C termini of the kinases (not shown), where PKA C subunits are placed in a well resolved group, clearly separated from other kinase families. On the contrary, a PrKX subfamily cannot be as clearly drawn by phylogenetic analysis only. Taking into account the experimental data provided herein, we would like to propose a more conserved PrKX subfamily including vertebrate and insect PrKX and a more extended family, which includes C. elegans (F47F2.1b), T. brucei (PKAC3), and possibly D. discoideum (DdpK2) (supplemental Fig. S1A). Unfortunately, all attempts to express full-length DdpK2 or an N-terminal deletion variant (Δ1–136) for interaction analyses in mammalian cells were unsuccessful (data not shown).

To address the PrKX family experimentally, we focused on its conserved R subunit inhibitory pattern, and we are able to provide evidence that human PrKX, mouse Pkare, D. melanogaster ΔDC2, and T. brucei PKAC3 all share the common feature of a RI over RII subunit preference with respect to autoregulation (Fig. 1). Human PrKγ, which lacks the εI-α loop, and a mutant C. elegans kinase (F47F2.1b, P325L), bind only to RII when tested with BRET (Fig. 1 and supplemental Fig. S2). By this, we conclude that many if not all kinases of the PrKX family are autoinhibited only by RI subunits, which contain a pseudosubstrate autoinhibitory domain. To substantiate these results, interaction analyses with homologous R subunits have to be performed. Recently, the D. melanogaster protein Swiss cheese was identified as a non-canonical R subunit binding to DC2, inhibiting both DC2 and the esterase activity of Swiss cheese mainly in the brain (44). Whether this regulation is conserved in mammalian tissues, awaits experimental evaluation, but this also points to the functional separation of conventional PKA and PrKX-like kinases.

**TABLE 2**

**Apparent activation constants (K_{act}) are reduced in mutant (CαL277R) holoenzymes**

| R subunit | K_{act} for cAMP (±S.D.) |
|-----------|--------------------------|
| RIα       | 105.5 ± 7                |
| RIIα      | 33.5 ± 0.7               |
| RIβ       | 118 ± 3                  |
| RIIβ      | 405 ± 14                 |
| CαL277R   | 55 ± 4                   |
| CαL277R   | 16 ± 1                   |
| CαL277R   | 53 ± 3                   |
| CαL277R   | 108 ± 16                 |

**FIGURE 4. Mutation of Cα subunit influences intracellular holoenzyme dynamics.** BRET analyses were carried out as detailed in the legend to Fig. 1. A: plasmids coding for GFP-Cα and GFP-CαL277R were co-transfected with constructs expressing either RIIα-Rluc or RIIαS99A-Rluc, β, as in A, combined with either RIβ-Rluc or RI1β14A-Rluc constructs. Depicted are original BRET values (mean ± S.E.) obtained from at least three independent repeats (n = 6 wells; *p < 0.01; **p < 0.005). + indicates treatment with forskolin/IBMX; −, indicates mock treatment. The dotted line represents the mean background value.
explained by the fact that Arg-245<sub>bRIi</sub> (Arg-241<sub>bRia</sub>) is involved in cAMP binding via Tyr-209<sub>bRIi</sub> (Glu-200<sub>bRia</sub>) (4, 50). Finally, Leu-277<sup>Cα</sup>C is involved in stabilizing the Arg-245/Asp-271<sub>bRia</sub> (Arg-241/Asp-267<sub>bRia</sub>) salt bridge, which breaks upon cAMP binding, followed by the major collapse of the α/C helix and subsequent holoenzyme dissociation (3, 4, 51). In summary, our work supports a critical involvement of the α-hα loop in autoregulation of PrKX-like kinases (Arg-283<sub>PrKX</sub>), in stabilizing PKA type II holoenzymes (Leu-277<sup>Cα</sup>C), and in proper allosteric signal propagation in PKA (Leu-277<sup>Cα</sup>C), besides the previously recognized role of this region in substate recognition (47, 48) and in stabilizing the active conformation of the kinase via the universally conserved Arg-280<sup>Cα</sup>C residue (40, 52).

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