PrKX Is a Novel Catalytic Subunit of the cAMP-dependent Protein Kinase Regulated by the Regulatory Subunit Type I*

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The human X chromosome-encoded protein kinase X (PrKX) belongs to the family of cAMP-dependent protein kinases. The catalytically active recombinant enzyme expressed in COS cells phosphorylates the heptapeptide Kemptide (LRRASLG) with a specific activity of 1.5 μmol/min·mg. Using surface plasmon resonance, high affinity interactions were demonstrated with the regulatory subunit type I (RI I a) of cAMP-dependent protein kinase (K_D = 10 nM) and the heat-stable protein kinase inhibitor (K_D = 15 nM), but not with the type II regulatory subunit (RII a, K_D = 2.3 μM) under physiological conditions. Kemptide and autophosphorylation activities of PrKX are strongly inhibited by the RI I subunit and by protein kinase inhibitor in vitro, but only weakly by the RII a subunit. The inhibition by the RI I subunit is reversed by addition of nanomolar concentrations of cAMP (K_a = 40 nM), thus demonstrating that PrKX is a novel, type I cAMP-dependent protein kinase that is activated at lower cAMP concentrations than the holoenzyme with the C_a subunit of cAMP-dependent protein kinase. Microinjection data clearly indicate that the type I R subunit but not type II binds to PrKX in vivo, preventing the translocation of PrKX to the nucleus in the absence of cAMP. The RII a subunit is an excellent substrate for PrKX and is phosphorylated in vitro in a cAMP-independent manner. We discuss how PrKX can modulate the cAMP-mediated signal transduction pathway by preferential binding to the RI I subunit and by phosphorylating the RI I a subunit in the absence of cAMP.

The cAMP-dependent protein kinases (cAPKs) play a key role in many signal transduction processes, mediating the majority of the known effects of cAMP in the eukaryotic cell. These multisubstrate enzymes regulate the activity of proteins involved in signal transduction, energy metabolism, cell proliferation, or differentiation by phosphorylation of Ser or Thr residues, which alters the biological properties of the target proteins (1–3). The activation of a hormone receptor that is linked by a heterotrimeric G-protein complex to adenylate cyclase leads to an increase in the concentration of the second messenger cAMP. Binding of cAMP activates the inactive cAPK holoenzyme tetramer by inducing dissociation of two monomeric catalytic (C) subunits from the regulatory (R) subunit dimer. The active C subunits are now able to phosphorylate target proteins in the cytosol or can translocate to the nucleus, activating cAMP-mediated gene expression (4). The presence of different isoforms of catalytic (C_a, C_b, and C_g) and regulatory (RI I a, RII p, RII_g, and RII I a) subunits suggests specific functions for these isoforms (5). However, preferential coexpression of any of these C subunits with either the type I or the type II R subunits has not been reported. The α and β isoforms of the catalytic subunit have nearly identical catalytic properties and a broad tissue distribution; C_a is generally the predominant form, whereas the C_g subunit is only found in testis (6). The two general isoforms of the R subunit, type I and type II, differ in molecular weight, tissue specificity, subcellular distribution, and expression pattern during development and cell cycle (2). The RI subunit is mainly found in the cytoplasm, whereas the RII subunit, which can bind to protein kinase A-anchoring proteins with high affinity, is localized in discrete particulate fractions in association with either membrane organelles or the cytoskeleton (7). The RII subunits contain a Ser at their autoinhibitory site and are readily autophosphorylated upon holoenzyme formation. The phosphorylation of this residue lowers the reassociation rate of the phosphorylated RII subunit with the released C subunit at least 5-fold (8). The decreased affinity prolongs the activation of the C subunit unless dephosphorylation of the RII subunit occurs (9). In contrast, the RI I subunit contains a pseudophosphorylation site, where the Ser is replaced by Ala and binds MgATP with high affinity (10, 11). Another class of specific cAPK inhibitors are the heat-stable protein kinase inhibitors (PKIs) (12, 13). These relatively small proteins bind with high affinity to the C subunit and favor the kinase activity of the R subunit, thereby blocking the cAMP response element-regulated gene expression (14).

The human protein kinase PrKX is related to the catalytic subunit of cAMP-dependent protein kinases (15) but is distinct from the isoforms C_a, C_b, and C_g. PrKX has 53.2% identity to the human C_a subunit of cAPK (PKA-C_a) in the catalytic core region. This degree of homology is much lower than the similarity of the two human isoforms C_a and C_g (90.5% identity). PrKX shows the highest sequence similarity to the DC2 protein kinase from Drosophila melanogaster (62.4% identity). DC2 is

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‡‡The abbreviations used are: cAPK, cAMP-dependent protein kinase; C, catalytic subunit; CREB, cAMP response element-binding protein; GST, glutathione S-transferase; MOPS, 3-(N-morpholino)propanesulfonic acid; PKA-C, catalytic subunit of cAMP-dependent protein kinase A; PKI, heat-stable inhibitor of cAMP-dependent protein kinase; PrKX, protein kinase X; R, regulatory subunit of cAMP-dependent protein kinase; RU, response unit(s); SPR, surface plasmon resonance; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; HA, hemagglutinin.
Type I cAMP-dependent Protein Kinase PrKX

A homologue of the major Drosophila catalytic subunit gene DC0 and can be inhibited by the heat-stable PKI and the regulatory subunit type I (16).

The catalytic core of PrKX is highly conserved, whereas the N terminus, which is important for stability and orienting subdomains in PKA-Cα (17), is completely different. PrKX mRNA is present in a variety of tissues, with the highest levels of expression in fetal and adult brain, kidney, and lung. Low levels of expression are also detected in adult placenta, heart, liver, skeletal muscle, and pancreas and in fetal liver (15). The gene PRKX is located on the human chromosomal subregion Xp22.3 and has a homologue called PRKY on the Y chromosome. An abnormal interchange between the X and the Y chromosomes that happens particularly frequently between PRKX and PRKY leads to the sex reversal disorder of XX males and XY females (18). This report focuses on the characterization of this novel protein kinase, PrKX, its interaction with known inhibitors of cAPK, and its regulation by the second messenger cAMP in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Cloning and Construction of PrKX Expression Vectors—The cDNA for PrKX was isolated from a random primed HeLa cell library (19). This cDNA differed from the original isolate described by Klink et al. (15) by a Val to Ala substitution at position 42. To facilitate purification of the recombinant protein, an N-terminal His6 tag (MGSSHHHHHHHHSSG) was added to the full-length construct, which was ligated into the StuI and HindIII sites of the SVX-Sport vector (Life Technologies, Inc.). High efficiency electrotransformation of this construct into COS cells resulted in overexpression of the PrKX protein. A GST-PrKX fusion protein was produced using the yeast expression system in Schizosaccharomyces pombe (Stratagene). A Smal/HindIII fragment was isolated from SVpKX and cloned into the StuI/HindIII sites of the HTb shuttle vector (Life Technologies, Inc.), a BamHI/HindIII fragment was isolated, and the HindIII site was filled in with Escherichia coli DNA polymerase Klenow fragment. This fragment was cloned into the BamHI/SmaI sites of ESP.

Expression and Purification of PrKX—PrKX expressed in COS cells was purified using Ni-nitrotriacetic acid affinity chromatography as described previously (19). Fractions containing the highest concentration of PrKX as determined by Coomassie staining of SDS-polyacrylamide gels were pooled, dialyzed against storage buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.25% Nonidet P-40, 20% glycerol, 1 mM dithiothreitol), and stored at −20 °C. GST-PrKX was overexpressed in S. pombe, purified using glutathione-agarose, and eluted with 10 mM glutathione (21).

Purification of PKA-Cα, RIα, RIβ, and GST-PKI—Wild-type recombinant PKA-Cα, was overexpressed in E. coli BL21 DE3 and purified by phosphocellulose chromatography as described previously (20). The fractions were further purified using Mono S chromatography (Amerham Pharmacia Biotech) (23). Recombinant RIα and RIβ subunits were overexpressed in E. coli 222, purified as described previously (24), and stored at −20 °C. To obtain cAMP-free R subunit, the R subunit was unfolded with 8 M urea and refolded as described by Buechler et al. (20) using Buffer D (20 mM MOPS, pH 7.0, 50 mM KCl, 1 mM ATP, 10 mM MgCl2, 1 mM dithiothreitol) at 30 °C. The Kd values for ATP and Kemptide were determined based on Michaelis-Menten kinetics, and data evaluation was performed using the software GraphPad Prism.

Kinetic Assays—The specific activity was determined using the spectrophotometric assay of Cook et al. (27) and the heptapeptide Kemptide (LRRASLG, Bachem) as substrate. The standard assay was performed at 22 °C in Buffer C (100 mM MOPS, pH 7.0, 1 mM ATP, 10 mM MgCl2, 1 mM dithiothreitol) with 250 μM Kemptide. Alternatively, activity was measured by a modified radioactive assay according to Roskoski (28) using Buffer D (20 mM MOPS, pH 7.0, 50 mM KCl, 1 mM ATP, 10 mM MgCl2, 1 mM dithiothreitol) at 30 °C. The Kd values for ATP and Kemptide were determined based on Michaelis-Menten kinetics, and data evaluation was performed using the software GraphPad Prism.

The effect of the free Mg2+ concentration on PrKX activity was examined using varying concentrations of Mg2+ and constant amounts of ATP and substrate. The free Mg2+ concentrations were calculated using the software Bound and Determined on equations provided by Brooks and Storey (29). The heat denaturation studies were performed according to Yonemoto et al. (30).

Autophosphorylation—1.2 μM PrKX was incubated in Buffer D with [γ-32P]ATP at 30 °C for 60 min. The inhibition of autophosphorylation was tested by adding a 2-fold molar excess of cAMP-free RIα or RIβ subunit in either the absence or presence of 20 μM cAMP; adding a 2-fold molar excess of GST-PKI or 20 mM EDTA; or heating PrKX at 60 °C for 5 min prior to the incubation. The autophosphorylation was stopped by adding SDS sample buffer. The samples were analyzed by SDS-PAGE and autoradiography.

Inhibition Studies with GST-PKI, RIα, and RIβ—60 nM PrKX was incubated with increasing amounts of GST-PKI and tested for activity with the spectrophotometric assay in Buffer C at 22 °C. The Kd values were determined using the equation of Cheng and Prusoff (31) based on Kd values determined by surface plasmon resonance (SPR). For inhibition studies with the regulatory subunits, 100 nM PrKX was incubated with increasing amounts of either cAMP-free RIα subunit or RIβ subunit in Buffer C at 22 °C and tested for Kemptide phosphorylation activity with the spectrophotometric assay.

Determination of Activation Constants (K0) for cAMP—Holoenzyme at a concentration of 5 nM formed by mixing PKA-Cα or PrKX and cAMP-free RIα, subunit in a 1:1.2 molar ratio in Buffer D was incubated for 2 min at room temperature with varying concentrations of cAMP between 1 nM and 2.5 μM. The reaction was started by adding 1 μM Kemptide, and the resulting phosphotransferase activity was measured using the radioactive assay.

SPR—SPR experiments were performed using a Biacore 2000 instrument (Biacore AB, Uppsala, Sweden). Here, the interaction between an immobilized component, referred to as the ligand, and a molecule in the mobile phase, the analyte, was determined. Changes in surface concentration are proportional to changes in the refractive index on the surface resulting in changes in the SPR signal, plotted as response units (RU). 1000 RU correspond to a surface concentration of 1 ng/mm2 (32). The association and dissociation of cAMP-free RIα was determined on a surface containing 580 RU of GST-PrKX immobilized via an oGST antibody at a flow rate of 30 μl/min in Buffer G. To determine the interaction between PKI and PrKX, 160 RU of GST-PKI were immobilized using the oGST antibody surface, whereas for the interaction with PKA-Cα, the immobilization level was reduced to 60–75 RU. A lower immobilization level of the ligand avoids rebinding effects in the dissociation phase that can arise due to fast on-rates. The regeneration of the antibody layer, i.e. the removal of the complete protein complex bound to the antibody, was performed using 10 μM glycine (pH 2.2) or 0.05% SDS and resulted in a completely regenerated and functional antibody surface. For the measurement of the interaction between PrKX or PKA-Cα and the RIα, subunit, the C subunits were covalently coupled to a CMS chip via primary amines (300 RU of His6-PrKX and 475 RU of PKA-Cα) as described (33), and the cAMP-free RIα subunit was injected at a flow rate of 10 μl/min in Buffer G or Buffer H. All buffers used contained 0.005% surfactant P20. The regeneration of the surface was performed in Buffer G with 100 μM cAMP and 2.5 mM EDTA. To determine unspecific binding, blank runs were performed on a control surface without ligand, and these values were subtracted. Kinetic constants were calculated by nonlinear regression of data using the Bioscience evaluation software, version 2.1 or 3.0 (Biacore). The association rate constant was calculated according to the equation,

\[
R = k_0 \text{CRM} \cdot (c + k_0) \cdot \left(1 - e^{-(c-k_0)t}\right) (\text{Eq. 1})
\]

where \(R\) is the SPR signal in response units, \(k_0\) is the association rate constant, \(k_0\) is the dissociation rate constant, and \(c\) is the concentration of the injected analyte at any time \(t\) during association. This equation describes the response at any time during association and can be used for nonlinear regression analysis of single curves. The dissociation rate constant was calculated according to the equation,

\[
R = R_0 e^{-k_d(t-t_0)} (\text{Eq. 2})
\]

where \(R_0\) is the response at time \(t\), and \(k_d\) is the rate at an arbitrary starting point \(t_0\). With the rate constants determined and known analyte concentrations, the equilibrium binding constants were calculated according to the following equation.

\[
k_0 = k_d/k_a (\text{Eq. 3})
\]

A 1:1 binding model assuming Langmuir conditions was applied to the data.

Phosphorylation of RIα Subunit—The phosphorylation of RIα (2 μM) by catalytic amounts of PrKX or PKA-Cα (2.4 nM) was performed with the radioactive assay at 30 °C. Aliquots of the phosphorylated RIα subunit were spotted onto phosphocellulose filters, washed three times in 0.5% α-phosphoric acid for 10 min, and counted using a liquid scintillation counter. In parallel, aliquots were analyzed by SDS-PAGE and

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autoradiography. Prephosphorylation of RIIa (2 μM) was performed with catalytic amounts of either PKA-Cα or PrKX (2.4 nM) in Buffer D using unlabeled ATP (1 mM). At certain time points, [γ-32P]ATP and the other kinase, PrKX or PKA-Cα, in the same concentration were added to the sample. The effect of prephosphorylation on 32P incorporation was demonstrated by SDS-PAGE and autoradiography.

Microinjection Experiments—10T1/2 mouse embryo fibroblasts were propagated in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 50% confluence and then microinjected with plasmids encoding HA-tagged PrKX and the mutant regulatory subunits RII(R209K) and RII(R213K), which are deficient in their response to cAMP. A contamination with endogenous PKA-Cα as indicated on the blot and 2 μg of His6-PrKX from COS cells after a wash with cAMP. A contamination with PKA-Cα in the preparation of PrKX could not be detected.

RESULTS

Expression, Purification, and Biochemical Characterization of PrKX—The expression of PrKX in eukaryotic cells (COS cells, S. pombe) yielded catalytically active protein kinase, which was used for the characterization. Initial preparations of PrKX overexpressed in bacteria were inactive and copurified with the bacterial chaperon GroEL. Inactivity and association with the chaperon most likely indicates folding problems, possibly due to a lack of posttranslational modifications, i.e. phosphorylation (34). Purification of PrKX was simplified by using fusion constructs with either an N-terminal His8 tag that can easily be purified with Ni-NTA affinity chromatography or a GST fusion tag (Fig. 1A). A wash step with cAMP (1 mM) was included in the purification strategy due to cross-contamination with endogenous PKA-Cα from COS cells, which was initially detected by Western blot analysis with a specific antibody against PKA-Cα and by using mass spectrometry. After this wash step, no contamination with endogenous PKA-Cα could be detected by Western blot analysis (Fig. 1B). The specific activity of PrKX determined either by the coupled spectrophotometric assay (27) or the more sensitive radioactive assay using the cPK standard substrate Kemptide was 1.5 ± 0.7 μmol/(min·mg), 20-fold less than the specific activity for PKA-Cα. The Km values for ATP (127 ± 9 μM) and Kemptide (58 ± 7 μM) were significantly higher compared with the wild-type or the His8-tagged PKA-Cα (Table I). PrKX phosphotransferase activity had an absolute requirement for Mg2+. PrKX displayed the highest activity at micromolar concentrations of free Mg2+.

Table I

| Specific activity | Km ATP | Km Kemptide |
|------------------|--------|-------------|
| His8-PrKX        | 1.5 ± 0.7 | 127 ± 9 | 58 ± 7 |
| Wild-type PKA-Cα | 30 ± 2 | 17 ± 5 | 20 ± 8 |
| His8-PKA-Cα      | 30 ± 3 | 14 ± 6 | 20 |

whereas higher concentrations of free Mg2+ had inhibitory effects on PrKX activity (Fig. 2A). PrKX was also autophosphorylated. Autophosphorylation was inhibited by the addition of EDTA (see Fig. 4), chelating the Mg2+. This result further confirmed the absolute requirement for Mg2+. Heat denaturation experiments were performed to determine the thermal stability of PrKX and yielded Tm values of 41.8 ± 0.2 °C for PrKX and 45.3 ± 0.2 °C for PKA-Cα (Fig. 2B). Thus, the thermostability of PrKX is only slightly reduced and comparable to PKA-Cα.

Regulation of PrKX Activity—The effects of physiological inhibitors of PKA-Cα (the RIα subunit, the RIβ subunit, and PKI) on PrKX were investigated. Both binding of these inhibitors and inhibition of phosphotransferase activity were quantified using different methods. The autophosphorylation of PrKX (Fig. 3) was significantly but not completely inhibited by a 2-fold molar excess of cAMP-free RIα subunit or GST-PKI. In contrast, the addition of a 2-fold molar excess of cAMP-free RIα subunit did not have any effect on PrKX autophosphorylation whether cAMP was present or not. Phosphotransferase activity of PrKX using Kemptide as substrate was significantly inhibited by both GST-PKI and the RIβ subunit. A 2-fold molar excess of PKI or RIβ subunit inhibited 80% of Tm 62 °C (Table I) and 95% (K = 1 μM) of this activity, respectively. Initial slopes of the inhibition curves indicated 1:1 stoichiometry. However, a 2-fold molar excess of RIα subunit did not lead to a detectable decrease of phosphotransferase activity (Fig. 4B). Addition of a 25-fold molar excess of RIγ subunit was necessary to cause a 40% inhibition, suggesting a much weaker inhibition by the RIγ subunit in the presence of ATP. This partial inhibition by the RIγ subunit could also be reversed by cAMP (Fig. 4C).

Activation by cAMP—The inhibition of PrKX activity by the RIα subunit of APK could be reversed by the addition of cAMP, as demonstrated in the autophosphorylation experiment (Fig. 3). To further examine the activation by cAMP, the PrKX/RIα holoenzyme was incubated with varying concentrations of this second messenger, and phosphorylation of Kemptide was determined. The activation constant (Ka) for the PrKX/RIα holoenzyme was 43 nM (Fig. 4D), which is 2.5-fold lower compared to the case of PKA-Cα.
His6-PrKX (fluence of Mg2+ detected by SDS-PAGE and autoradiography, was inhibited by the phosphorylation of PrKX (a are proteolytic fragments of the RII subunit). Assay.

For 3 min. The remaining phosphotransferase activity was determined carried out in 20 mM MOPS, 150 mM KCl, 1 mM dithiothreitol, pH 7.0, with the free Mg2+ concentrations, calculated according to Brooks and Storey (29), were varied as indicated. Autophosphorylation (PrKX for 5 min at 60 °C prior to the incubation completely abolished with the RII subunit. The RII (lane 6) was phosphorylated by PrKX (lane 3). The RII subunit was reversed by cAMP (100 μM) (Fig. 3). Hill constants were determined for PrKX and PKA-Cα, respectively (Fig. 5). Regeneration of the surface was performed with CAMP. The equilibrium binding constants (KD) for the His6-tagged and GST-PrKX were 5.4 and 4.9 nM, respectively, which suggests that the different fusion tags do not influence the affinity to the RII subunit. However, the association and the dissociation rate constants were affected slightly (Table II). Comparable rate constants for the interaction between RII subunit and either amine-coupled His6-tagged or wild-type PKA-Cα indicated that the N-terminal His6 tag also did not change the affinity to the RII subunit (Table II). Only GST-PKACα showed a slight difference, with a 2-fold lower dissociation rate constant.

PKI was immobilized site-direct ed via the GST fusion tag on an αGST antibody surface. Because no physiological dissociation mechanism is known, the protein complex was completely removed from the antibody surface after each interaction analysis and subsequently reimmobilized with GST-PKI at the same concentration level. KD values of 15 and 0.5 nM were determined for PrKX and PKA-Cα, respectively, in the presence of 1 mM ATP/10 mM MgCl2 (Fig. 6). The association rate constant for PrKX was 4-fold lower, and the dissociation rate constant was 7.5-fold faster than for PKA-Cα (Table III).

Initial data suggested that the PrKX/RIIα subunit interaction is dependent on the absence of MgATP. This interaction was measured both in the absence and in the presence of MgATP using again a variety of immobilization strategies and fusion tags. Similar binding constants were obtained.2 In the absence of MgATP, the KD value for the PrKX/RIIα interaction was 43 nM. However, in the presence of MgATP, the KD value increased dramatically to 2.3 μM (Fig. 7, C and D; Table IV). In comparison, the KD values for the PKA-Cα/RIIα interaction were 0.1 nM in the absence and 0.7 nM in the presence of MgATP (Fig. 7, A and B; Table IV).

Phosphorylation of RIIα Subunit—The results of the autophosphorylation assay (Fig. 3) demonstrated that the RIIα subunit is an excellent substrate for PrKX. To determine the rate of phosphorylation of the RIIα subunit 2 μM RIIα subunit were phosphorylated with catalytic amounts (2.4 nM) of either PrKX or PKA-Cα in the absence or in the presence of 50 μM CAMP. In the presence of CAMP, there was a 32P incorporation of 0.95 mol/mol and 0.5 mol/mol after 20 min using PKA-Cα and PrKX, respectively (Fig. 8A). Using higher concentrations of both C subunits, a stoichiometry of about 1 mol/mol was achieved, suggesting that a single residue is phosphorylated by both C subunits.3 The initial slope of the phosphorylation kinetic was used to determine the specific activity when using RIIα subunit as a substrate (Fig. 8A). In the absence of CAMP, there was only low phosphorylation of RIIα by PKA-Cα (0.07 ± 0.01 μmol/(min·mg)). However, upon the addition of CAMP, there was an 18-fold increase in activity (1.25 ± 0.08 μmol/(min·mg)). In contrast, the RIIα subunit was phosphorylated by PrKX with a specific activity of 0.36 ± 0.06 μmol/min·mg in a CAMP-independent manner, which is 3.5-fold lower than the phosphorylation by PKA-Cα in the absence of CAMP. To determine whether PKA-Cα and PrKX phosphorylate the same residue on the RIIα subunit, the RIIα subunit was pre-
phosphorylated by either PKA-Cα and PrKX using unlabeled ATP. The phosphorylated RIIα was then used as a substrate in a second phosphorylation experiment containing radiolabeled ATP and the other kinase. In both cases, prephosphorylation led to a significant decrease of 32P incorporation, indicating that PKA-Cα and PrKX phosphorylate the same residue (Fig. 8B).

In Vivo Interaction in Microinjected Cells—Plasmids encoding HA-tagged PrKX were microinjected in mouse embryo 10T1/2 fibroblasts. PrKX expressed in vivo localized to the cytoplasm, but addition of the membrane-permeable dibutyryl-cAMP to the microinjected cells induced a translocation of PrKX into the nucleus within about 15 min (Fig. 9). This suggests that PrKX interacts with endogenous regulatory subunit of cAMP-dependent protein kinase present in the cytoplasm and dissociates from the regulatory subunits in response to cAMP. To clarify whether the cytoplasmic localization is due to interaction with the regulatory subunit type I or type II, coinjections with plasmids encoding mutant R subunits deficient in their response to cAMP were performed. The coexpression of PrKX with RIα(R209K) shows a cytoplasmic localization of PrKX in the absence and in the presence of cAMP, whereas the coexpression with the corresponding mutant RIIα(R213K) shows the same localization pattern as for PrKX alone (Fig. 9, A–C). These in vivo experiments clearly demonstrate that the regulatory subunit type I is the interacting regulatory protein for PrKX, whereas the mutant RIIα subunit is not able to keep PrKX in the cytosol.

DISCUSSION

Based on sequence alignments with the catalytic subunits of cAPKs from several organisms, the novel human protein kinase PrKX is predicted to belong to the family of cAMP-dependent protein kinases. Comparison of primary sequences showed conservation of many residues that have been demonstrated to be important for substrate recognition and the interaction with the regulatory subunits type I and type II and PKI in PKA-Cα. This includes residues that are important for binding the substrate consensus sequence, RRX(S/T)Y, i.e., the binding site for the P-2 Arg (Glu-170 and Glu-230), the P-3 Arg (Glu-127 and Asp-328) and the P+1 (Leu-198, Pro-202, and Leu-205) (35), but are not conserved throughout the protein kinase family (36). There is only one conservative change, from Asp-328 to Glu in PrKX. The P-site and Y are referred to as the phosphorylation site and a hydrophobic residue, respectively. Therefore, it is likely that the substrate recognition sequences of
PrKX and PKA-Cα are similar, and Kemptide, the standard cPK peptide substrate (LRRASLG), can be used as substrate for PrKX. Despite higher $K_m$ values and a 20-fold lower specific activity compared with PKA-Cα, the kinetic properties of PrKX from COS cells still indicate that PrKX is a PKA-like kinase. The phosphotransferase activity of PrKX has an absolute requirement for Mg$^{2+}$ but is reduced in the presence of millimolar concentrations of free Mg$^{2+}$, suggesting that PrKX also has two metal binding sites, one activating and one inhibiting site, similar to PKA-Cα (37).

Several lines of evidence demonstrate that PrKX is a novel catalytic subunit of the cAMP-dependent protein kinase: 1) autophosphorylation and Kemptide phosphorylation of PrKX could strongly be inhibited by the RIα subunit. The inactive holoenzyme consisting of the RIα subunit and PrKX was activated at 2.5-fold lower concentrations of cAMP compared with the holoenzyme consisting of RIα and PKA-Cα. This increased sensitivity to cAMP was also found for the holoenzyme containing the neural form of the RI subunit, RIβ (38) and suggests an earlier response to cAMP. 2) Binding of both R subunits, determined by SPR as described under “Results,” can be reversed completely by the addition of cAMP. 3) Microinjection with plasmids encoding PrKX showed a nuclear translocation in response to a membrane permeable analog of cAMP, dibutyryl-cAMP, indicating that PrKX expressed in mouse embryo 10T1/2 fibroblasts is inhibited by endogenous R subunit in the absence of cAMP. In the presence of cAMP, PrKX is released and can translocate to the nucleus.

A major difference from PKA-Cα is the regulation by the R subunits. Autophosphorylation of PrKX was significantly inhibited by the RIα subunit but was not affected by the RIβ subunit in the presence of ATP. Kemptide phosphorylation by PrKX was inhibited stoichiometrically by the RIβ subunit but only by a large molar excess of the RIβ subunit. This suggests a much lower affinity to the RIβ subunit in the presence of physiological concentrations of MgATP. Using SPR, these data

\[
\text{GST-PrKX / RIα} \\
\begin{array}{ccc}
454nM & 890nM & 223nM \\
111nM & 56nM & 29nM \\
\end{array}
\]

FIG. 5. High affinity binding of GST-PrKX to the RIα subunit determined by SPR. GST-PrKX was immobilized to an αGST-antibody surface as described under “Experimental Procedures,” and cAMP-free RIα subunit in the concentrations indicated on the plot was injected in the presence of ATP/Mg$^{2+}$. The association and dissociation phases were monitored for 300 s by following the change in SPR signal, given in RU. Association and dissociation phases are indicated on the plot.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Subunit & $K_d$ & $K_d$ & $K_d$ \\
\hline
His$_α$-PrKX & $2.6 \times 10^5$ & $1.4 \times 10^{-3}$ & 5.4 \\
GST-PrKX & $1.1 \times 10^6$ & $5.4 \times 10^{-4}$ & 4.9 \\
His$_α$-PKA-Cα & $5.5 \times 10^9$ & $2.5 \times 10^{-4}$ & 0.45 \\
Wild-type PKA-Cα & $4.5 \times 10^5$ & $3 \times 10^{-4}$ & 0.67 \\
GST-PKA-Cα & $3.9 \times 10^5$ & $1.5 \times 10^{-4}$ & 0.38 \\
\hline
\end{tabular}
\caption{Rate and affinity constants for the interaction of PrKX and PKA-Cα using SPR}
\end{table}

\[
\text{GST-PKI / PrKX} \\
\begin{array}{ccc}
96nM & 48nM & 24nM \\
12nM & 6nM & 3nM \\
\end{array}
\]

FIG. 6. PKI binds with high affinity to His$_α$-PrKX and PKA-Cα. GST-PKI was immobilized to an αGST antibody surface as described under “Experimental Procedures.” The association and dissociation phases of His$_α$-PrKX (A) and PKA-Cα (B) injected in the presence of ATP/Mg$^{2+}$ were monitored by following the change in SPR signal, given in RU. The concentrations of the analytes are given on the plots.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Subunit & $K_d$ & $K_d$ & $K_d$ \\
\hline
His$_α$-PrKX & $3.8 \times 10^5$ & $5.8 \times 10^{-3}$ & 15 \\
Wild-type PKA-Cα & $1.5 \times 10^6$ & $7.6 \times 10^{-4}$ & 0.5 \\
\hline
\end{tabular}
\caption{Rate and affinity constants for the interaction of PrKX and PKA-Cα with PKI using SPR}
\end{table}
were confirmed and compared with the interaction of PKA-C\(_{\alpha}\). The \(K_D\) value for PKKX and the RI\(_{\alpha}\) subunit determined in the presence of ATP is 5 nM, whereas the corresponding value for PKA-C\(_{\alpha}\) is in the subnanomolar range and corresponds well with the value reported by Hofmann (10). The affinity of PKKX for the RII\(_{\alpha}\) subunit is strongly dependent on MgATP. In the absence of MgATP, the affinity to RII\(_{\alpha}\) \((K_D = 43\text{ nM})\) was 430-fold less than for PKA-C\(_{\alpha}\). In the presence of physiological concentrations of MgATP, the affinity decreased to a \(K_D\) of 2.3 \(\mu\)M, which is almost 2 orders of magnitude lower. In contrast, the decrease in affinity was only 7-fold in the case of PKA-C\(_{\alpha}\) (Table IV). Comparing now the \(K_D\) values between PKA-C\(_{\alpha}\) and PrKX in the presence of MgATP, a 3300-fold difference was observed. These data imply that under physiological conditions the RII\(_{\alpha}\) subunit is a potent inhibitor for PKA-C\(_{\alpha}\) but not for PrKX.

Coinjection of DNA encoding PrKX with DNA encoding mutant forms of the RI and the RII subunit further demonstrated that only the RI subunit, not the RII subunit, is able to keep PrKX in the cytosol. Both mutant proteins, RI(R209K) and RII(R213K), are deficient in cAMP binding to the cAMP binding site A (39). RI(R209K) mutant protein does not respond to dibutyryl-cAMP at the concentrations used, preventing the release of PrKX as shown in Fig. 9E. If the cells are injected with a plasmid encoding the RII(R213K) mutant protein, the same concentration of dibutyryl-cAMP causes a translocation of PrKX to the nucleus, indicating that PrKX is kept in the cytosol, most likely by the endogenous RI subunit, and is not anchored by the cAMP-insensitive mutant RII subunit.

Inhibitors of cAPK, such as PKI and the RI subunit, contain pseudo-substrate sites, whereas the RII subunit is a substrate of PKA-C\(_{\alpha}\) that inhibits with a subnanomolar binding constant (10). The phosphorylation of the RII\(_{\alpha}\) subunit in the presence of cAMP leads to an at least 5-fold reduction in the affinity for PKA-C\(_{\alpha}\) (8), which is in excellent agreement with the SPR studies presented here. PrKX also phosphorylates the RII\(_{\alpha}\)
subunit, but neither autophosphorylation nor Kemptide phosphorylation is significantly inhibited by RIIα subunit. Thus, PrKX phosphorylates the RIIα subunit in a cAMP-independent manner, whereas PKA-Cα in catalytic amounts phosphorylates the RIIα subunit significantly only in the presence of this second messenger. The phosphorylation of the RIIα subunit occurs at the autoinhibitory site, and PrKX most likely phosphorylates the same residue as PKA-Cα, as demonstrated by the prephosphorylation assay. Because the phosphorylation of the RIIα subunit lowers the reassociation rate of the type II holoenzyme, PrKX could modulate the activation state of PKA when its catalytic subunit associates with the RIIα subunit.

Another important role of the regulatory subunits type I and II is to target the C subunits to distinct subcellular compartments. The RIIα subunit serves as a cytoplasmic anchor for the C subunit, as well as for PrKX, as demonstrated here, whereas the RIIβ subunit keeps the C subunit at specific organelles via specific protein kinase A-anchoring proteins (7). A dual-specificity protein kinase A-anchoring protein that binds both RI and RII subunits has been recently described (40). In this way, the RI and the RII subunits may serve to localize the C subunit close to relevant substrates or local pools of cAMP, providing distinct functions for these isoforms. Indeed, it has been suggested that the type I isoform is a positive effector of growth, and the type II isoform relates more to tissue differentiation (2). Besides the possibility that PrKX as a type I cAPK might have a specific function in this pathway itself, the alteration of the affinity for one of the regulatory subunits, i.e., by phosphorylation of the RIIα subunit by PrKX, might change the balance between both isoforms or the subcellular localization of PKA-Cα. Therefore, PrKX may act to modulate the distinct functions of these isoforms.

Although the crystal structure of the holoenzyme has not been solved, some important features for the interaction between the C and R subunits have been determined. In addition to the pseudosubstrate site, two basic residues (Lys-213 and Lys-217) have been demonstrated to be essential for holoenzyme formation with the RIIα subunit (41). Both are conserved in PrKX, with only a conservative change from Lys-217 to Arg. The N terminus of PKA-Cα contains a myristylation motif and a long amphipathic helix (A-helix), which is important for the structural integrity and the correct orientation of subdomains at the cleft interface. Mutations in the N terminus strongly affect the activation of the RIIα but not the RIα holoenzyme (17). The N terminus of PrKX is completely different from that of PKA-Cα and lacks both the myristylation motif and the long amphipathic helix. However, PrKX has a proline-rich motif that might be a putative binding site for SH3 or WW domains. Previous work has demonstrated that SH3 domains recognize sequences containing proline and hydrophobic residues (42), and it is already clear that the protein-protein interactions mediated by these domains play an important role in the control of different signaling pathways (43). The WW domain is a protein module present in a number of signaling and regulatory proteins and is known to be a binding site for proline-rich peptides (44). The differences in the N terminus, the preferential binding of PrKX to one of the regulatory subunits, and the phosphorylation of the RIIα subunit are important features that distinguish PrKX from PKA-Cα not only in structure but also in subcellular localization and involvement in other signal transduction pathways in the eukaryotic cell.

PKI, a specific inhibitor of PKA-Cα (12), also interacts with
CREBs at Ser-133, and this transcriptional activation is rate-of Rep expression. The interaction of gene expression induced by cAMP response element-binding proteins (CREBs). A number of eukaryotic genes are regulated by the PKA-Cα-mediated phosphorylation of CREBs at Ser-133, and this transcriptional activation is rate-limited by the nuclear export of PKA-Cα (14, 46), thus regulating the transcriptional activation of gene expression induced by cAMP response element-binding proteins (CREBs). A number of eukaryotic genes are regulated by the PKA-Cα-mediated phosphorylation of CREBs at Ser-133, and this transcriptional activation is rate-limited by the nuclear export of PKA-Cα (47). PrKX has a putative nuclear localization signal (48) in the C terminus (RRXXKHHR) and thus might also be involved in the regulation of gene expression, but the subcellular localization and in vivo function of PrKX remains to be elucidated.

A novel class of inhibitors for the cAMP-dependent protein kinases PKA-Cα and PrKX are the viral nonstructural proteins of adeno-associated virus type 2 Rep78/52. Infection of primary human cells with adeno-associated virus type 2 and overexpression of the Rep proteins lead to a decrease in cellular proliferation and to growth arrest (49). In infected cells, the viral Rep proteins might affect the activity of both kinases or its regulation by CAMP. In addition, the subcellular localization mediated by the interaction with the regulatory subunits, RI and RII, and the transcriptional regulation by CREB phosphorylation might also be influenced. The mechanism of Rep-mediated inhibition of cell growth has not been established yet, but the inhibition of PKA-Cα and PrKX by Rep78/52, as demonstrated by Chiorini et al. (19) may explain some of the effects of Rep expression.

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