The catalytic (C) subunit of cAMP-dependent protein kinase is inhibited by the regulatory (R) subunit and by a thermostable inhibitor (PKI). Both inhibitors also affect the intracellular distribution of the C subunit. Whether injected into the cytoplasm or into the nucleus, free C subunit can enter and exit the nucleus freely. After 30 min its distribution is identical and is independent of the initial site of injection. In contrast, when C is injected into the cytoplasm, whether it is complexed with R or PKI, the complexes are restricted to the cytoplasm (1–3). However, unlike the R subunit, which is restricted to the cytoplasm like the holoenzyme, free PKI enters the nucleus rapidly following its injection into the cytoplasm. When holoenzyme is injected directly into the nucleus, it cannot exit and return to the cytoplasm. In contrast, nuclear injection of a C–PKI complex results in the rapid exit of the C subunit from the nucleus. In equilibrated cells previously injected with the C subunit, subsequent cytoplasmic injection of either PKI or type 1 R depletes the nucleus of C although PKI does so faster, consistent with its ability to restrict the access of the C subunit to the nucleus. In equilibrated cells previously injected with the C subunit, subsequent cytoplasmic injection of either PKI or type 1 R depletes the nucleus of C although PKI does so faster, consistent with its ability to restrict the access of the C subunit to the nucleus. In equilibrated cells previously injected with the C subunit, subsequent cytoplasmic injection of either PKI or type 1 R depletes the nucleus of C although PKI does so faster, consistent with its ability to restrict the access of the C subunit to the nucleus.

The cAMP-dependent protein kinase contains both regulatory (R) and catalytic (C) subunits, and the mechanism by which the enzyme is activated involves subunit dissociation. In the absence of cAMP, the enzyme exists as an inactive tetrameric holoenzyme (R2C2). Upon binding of cAMP to R, the holoenzyme dissociates into an R2 dimer and two monomeric, catalytically active C subunits that phosphorylate protein substrates (for reviews, see Refs. 4–6). The dissociated C subunit not only acts on cytoplasmic proteins but also enters the nucleus where it modifies nuclear substrates such as the transcription factor cAMP response element-binding protein (CREB) (7). Phosphorylation of CREB by the C subunit is required for the transcriptional activation of genes containing CRE-regulated promoters (8). Because the effects of the C subunit are pleiotropic, its activity must be strictly regulated. In addition to reversible inactivation by the R subunit (9), the monomeric C subunit is also inhibited by the naturally occurring thermostable protein kinase inhibitor (PKI) (10) for a review, see Ref. 11). Inhibition by PKI occurs specifically in the presence of cAMP when the C subunit is dissociated from its holoenzyme complex. Despite substantial differences in their structures, R and PKI bind and inhibit C by analogous mechanisms. Each contains an inhibitory sequence that resembles a peptide substrate, hence binding is competitive with respect to other substrates (for a review, see Ref. 5). Consequently, the binding of R and PKI are mutually exclusive. Despite being identified more than 20 years ago (12), the function of PKI in vivo remains obscure. In an attempt to distinguish the role of R from that of PKI in the attenuation of cAMP-mediated cellular responses, we examined their effects on the intracellular trafficking and biological activity of the dissociated C subunit. In previous studies, we demonstrated that a proportion of the fluorescently labeled C subunit localized rapidly in nuclei following its microinjection into the cytoplasm of rat fibroblasts (1). In contrast, the labeled type 1 R subunit (R1) or the type I holoenzyme (R2C2) labeled in either subunit remained in the cytoplasm. Fluorescence originating from the C subunit in holoenzyme was detected in the nucleus only after treating the cells with agonists such as 8-bromo-cAMP (1), forskolin, or prostaglandin E1 (2), agents that cause holoenzyme dissociation. Following removal of agonist, labeled subunits reassociated in the cytoplasm as evidenced by reconstitution of fluorescence resonance energy transfer (2). Thus the R1 subunit functions not only as an enzymatic inhibitor of C but also as a cytoplasmic anchor of the C subunit, while free C can enter the nucleus. Similarly, the injection of catalytically inactive C–PKI complexes resulted in exclusively cytoplasmic fluorescence (3). Loss of enzymatic activity alone was not sufficient to block the nuclear localization of the free C subunit (3). Hence association with either R1 or PKI prevents the nuclear localization of C. We now report that both inhibitors are capable of depleting the nucleus of the dissociated C subunit, although
at different rates and probably by different mechanisms. Moreover, both inhibitors significantly reduce changes in gene expression mediated by C. Unlike R, however, PKI rapidly enters the nucleus following its injection into the cytoplasm. Nuclear microinjection of C-PKI complexes results in the enhanced export of C from the nucleus. Together, these results suggest a novel mechanism for the regulation of signal transduction through the C subunit, implying a potential physiological role for PKI in excluding the C subunit from the nucleus and returning it to the cytoplasm.

EXPERIMENTAL PROCEDURES

Materials—Fluorescein 5-isothiocyanate (isomer “T”) (FITC), tetramethylrhodamine isothiocyanate (TRITC), and 5-iodoacetamido-fluorescein (IAF) were obtained from Molecular Probes (Eugene, OR). Kemptide (LRRASLG) was synthesized by the Peptide/Oligonucleotide Facility, University of California, San Diego. All other chemicals were of reagent grade or the highest quality available commercially. Centricon microconcentrators were obtained from Amicon.

Purification and Labeling of Proteins—For localization studies, an expression vector encoding a modified form of PKI engineered to include tandem cysteine residues at its COOH terminus (PKIcys) (kindly provided by P. Howard and R. Mauer, University of Iowa) was expressed. PKIcys was purified as described previously for wild type recombinant PKIα (13). Sulphydryl groups were labeled with 5-IAF. To 1 mg of PKIcys in 1 ml of 100 mM Hepes pH 8.0 was added β-mercaptoethanol to a molar ratio of 2:1 over cysteine residues. Following incubation at room temperature for 10 min, the labeling reaction was initiated by adding the fluorophore to a 5:1 molar ratio relative to total sulphydricys. After incubation under N2 in the dark at 70 °C for 30 min, the reaction was terminated by the addition of a 50-fold molar excess of β-mercaptoethanol over the fluor. Products were separated by exclusion chromatography (Sephadex G-25 equilibrated with water) at room temperature, and an aliquot of the eluant was analyzed by titration to confirm that the potency of the labeled PKI was comparable with that of unlabeled PKI. The purification was examined by denaturing gel electrophoresis to ensure the absence of unincorporated fluorophore. The sample was filtered, concentrated by lyophilization, and quantitated by titration against C before injection. Recombinant PKIα was purified as described (13) and quantitated by titration against the C subunit. The α subunit was expressed in Escherichia coli (14) and purified as described (15). The recombinant mammalian catalytic subunit was purified from E. coli as described (16), followed by fast protein liquid chromatography (17). The protein was labeled with FITC as described previously (1) and exchanged into injection buffer (5 mM sodium phosphate, pH 7.4/100 mM KCl); the final concentration of the catalytic subunit ranged from 8-10 mg/ml/mL. Activity was measured with a synthetic peptide, Kemptide, with a coupled spectrophotometric method (18). When analyzed by Coomassie Blue staining following polyacrylamide gel electrophoresis carried out in the presence of SDS, the protein appeared as a single band. Coincident fluorescence was detected by irradiating the gel at 300 nm. To prepare the C-PKI complex, purified 32P-labeled FITC subunit was mixed with a 2-fold molar excess of unlabeled recombinant PKIα. Following complexation, an aliquot of the mixture was assayed and verified to be devoid of enzymatic activity. To prepare the C-PKI(6–20)–PKI(58–200) complex, purified 32P-labeled FITC subunit was mixed with a 60-fold molar excess of PKI(58–200) as described previously (3). No catalytic activity could be observed in this mixture. In addition to the wild type PKIα, a mutant was prepared where Arg-18 and Arg-19 at the consensus site were replaced by Ala. This mutant PKI was isolated using a procedure identical to the one used for PKIα. The C-mutant PKI (R18A,R19A) complex was prepared in the same way as C-PKI using a 2-fold molar excess of the mutant PKI. Activity was not inhibited under these conditions.

Cells and Microinjection—REF52 cells were propagated and injected as described previously (3). Following injection, the cells were fixed in 3.7% formaldehyde/phosphate-buffered saline for 5 min at room temperature. Where indicated, the cells were stained with TRITC-conjugated anti-rabbit antibody (15 μg/ml) (Jackson Laboratories) in phosphate-buffered saline (PBS)/10% Nonidet P-40/1 mg/ml bovine serum albumin for 20 min at room temperature. The cells were observed and photographed using a Zeiss axiophot fluorescence microscope and a × 40 (1.3 NA) objective. Photomicrographs were made using TMY film (ASA 800 or 1600).

Quantitative Microscopy and Photobleaching—Cells were imaged on a Zeiss Axiovert microscope using a ×40 (1.3 NA) objective (Nikon CF) and a cooled CCD camera (Photometrics, Tucson, AZ) interfaced to a Silicon Graphics Personal Iris. For most experiments, intensities were averaged over roughly circular 10-μm diameter regions of interest in the nucleus or cytoplasm. When nuclear to cytoplasmic ratios were calculated, an area of the cytoplasm adjacent to the nucleus was selected to have roughly the same local thickness. The cytoplasm of cells was photobleached by closing down the field stop of the microscope to illuminate a spot of 30-μm diameter and increasing the illumination power by a factor of 32 for 10 s. Typically 50% of the FITC-labeled protein in the area of illumination was photobleached. Photobleaching recoveries were expressed as a ratio of the intensity from a bleached to a nonbleached region of the cell to compensate for any residual photobleaching that occurred during data acquisition.

RESULTS

PKI Localizes to the Nucleus—Various studies have examined the subcellular localization of the subunits of cAMP-dependent protein kinase (Refs. 1, 2, 19, and 22 for reviews). Most recently we demonstrated that PKI, when injected as a preformed C-PKI complex into the cytoplasm, is functionally equivalent to the R subunit in preventing nuclear localization of the C subunit (3). This effect of PKI prompted investigation of its subcellular distribution when it was not complexed with the C subunit. Two forms of PKI were evaluated: wild type recombinant PKIα and a modified form engineered to include tandem cysteine residues (PKIcys). Recombinant PKIα was labeled with FITC or TRITC, and PKIcys was labeled with IAF. Following injection into the cytoplasm of rat fibroblasts, all preparations of labeled PKI readily entered the nucleus (modified form shown in Fig. 1). Significant nuclear fluorescence was observed when the cells were analyzed as early as 5 min or as late as 60 min following PKI injection. Nuclear fluorescence was also observed in unfixed living cells (not shown). Similar results were observed in three independent cell lines, REF52, rat2, and B/c3T3 fibroblasts (not shown). Thus, although both PKI and the R subunit are capable of inhibiting the C subunit and preventing its entry into the nucleus, the inhibitor proteins differ in their own capability of accessing the nucleus. The large R subunits, based on microinjection studies, are restricted to the cytoplasm as is the holoenzyme. In contrast, the small inhibitor, PKI, which contains only 75 amino acids, localizes rapidly to the nucleus when it is not complexed with the C subunit.

Fig. 1. PKI accumulates in the nucleus. IAF-labeled PKI(200 μM) was injected into the cytoplasm of exponentially growing REF52 cells. At various times after injection (30 min, shown here) the cells were fixed, and the distribution of PKI was examined by fluorescence microscopy. IAF-PKI entered the nucleus as did 32P-PKIα and 32P-PKIα (not shown).
PKI Enhances the Nuclear Export of the C Subunit

Cytoplasmic Injection of PKI into Cells Previously Injected with the C Subunit Alters the Nuclear Distribution of the C Subunit—To examine the ability of PKI to alter the subcellular distribution of the C subunit, a series of sequential injection experiments were performed. In these experiments, the FITC-labeled C subunit (FITC) was first injected into the cytoplasm, and the cells were incubated for 30 min to allow the C subunit to enter the nucleus (Fig. 2a). Subsequently, a mixture containing affinity-purified rabbit IgG (as a marker) and PKI was injected into the cytoplasm of the cells previously injected with the FITC subunit to see if PKI would enter the nucleus and return the C subunit to the cytoplasm. At various times after secondary injection, the cells were fixed and stained with a TRITC-conjugated anti-rabbit antibody to identify those cells receiving the IgG/PKI mixture. As it was not possible to reinject all cells, each field of cells contained those injected only with FITC, those injected only with IgG/PKI, and those injected with both FITC and IgG/PKI.

Fig. 2. Cytoplasmic injection of PKI alters the nuclear localization of the FITC subunit. Panels a and b display fields of REF52 cells fixed at 30 min (a) or 2.5 h (b) following cytoplasmic injection of FITC (60 μm). Panels c, e, and g display the localization of FITC at 5 (c), 15 (e), or 30 min (g) after secondary injection of IgG/PKI (2 mg/ml IgG/250 μM PKI). Cells receiving both FITC and PKI are designated with arrows in panels c, e, and g. Panels d, f, and h exhibit the same fields of cells stained for coinjected IgG/PKI. Panels c, e, and g correspond to panels d, f, and h, respectively.
PKI Enhances the Nuclear Export of the C Subunit

The distribution of the F'TCC subunit was examined in these dually injected cells as a function of time. The C subunit equilibrated between the nucleus and cytoplasm within 30 min of injection (Fig. 2a), and its distribution in the absence of PKI remained unchanged for as long as 2.5 h (Fig. 2b or cells in 2e, 2g lacking arrows). Cells receiving both F'TCC and IgG/PKI still retained the C subunit mostly in the nucleus 5 min following PKI injection (Fig. 2c, arrowed cells). Within 15 min after PKI injection, however, the nuclear fluorescence of C was significantly depleted in most cells (Fig. 2e, arrowed cells) and now appeared in the cytoplasm. Within 30 min of PKI injection, most dually injected cells exhibited predominantly cytoplasmic fluorescence emanating from the F'TCC subunit (Fig. 2g, arrowed cells). Injection of F'TCC followed by injection of IgG alone had no effect on the distribution of the F'TCC subunit (data not shown).

To assess if other proteins that accumulate in the nucleus might also affect the distribution of the C subunit, the F'TCC subunit was injected, and 30 min later the same fields of cells were reinjected with a mixture containing IgG and soybean trypsin inhibitor (STI). STI was chosen because, like PKI, it is a small protein that accumulates in the nucleus following microinjection (23). The molar ratio of C:STI was the same as that for C:PKI in the previous experiment. Cells dually injected with F'TCC and IgG/STI exhibited largely nuclear fluorescence emanating from F'TCC for as long as 60 min following injection of IgG/STI (Fig. 3c, arrowed cells), unlike those cells dually injected with F'TCC and IgG/PKI in the same experiment (Fig. 3a, arrowed cells).

A Dominant Negative Regulatory Subunit Can Also Retrieve the C Subunit from the Nucleus—To determine if other molecules that bind the C subunit were capable of altering its intracellular distribution, analogous experiments were performed using various preparations of R', including R'(R209K) (24). This dominant negative variant contains a mutation in CAMP-binding site "A", raising the K for cAMP so that the resulting holoenzyme is very resistant to activation by cAMP (25). In vitro this R' subunit binds instantaneously to the C subunit, thus inhibiting its activity. The F'TCC subunit was injected into the cytoplasm, and 30 min later, the same fields of cells were reinjected into the cytoplasm with a mixture of IgG and R'(R209K). The molar ratio of R'(R209K):F'TCC was equal to the PKI:F'TCC ratio used in the previous experiments. Unlike cells injected sequentially with F'TCC and then PKI, cells injected with F'TCC and then R'(R209K) exhibited largely nuclear fluorescence 15 min after injection of the regulatory subunit (Fig. 4a, arrowed cells). The nuclear fluorescence was depleted, however, in some cells analyzed 30 min after R'(R209K) injection (Fig. 4c, arrowed cells) and largely absent from many cells 60 min after injection (Fig. 4e, arrowed cells). Thus, although qualitatively similar to dual injections with PKI, more time was required for depletion of nuclear fluores-

![Fig. 3. STI does not alter the nuclear distribution of the C subunit. REF52 cells were injected with F'TCC (60 μM), incubated for 30 min, and then reinjected with rabbit IgG (2.0 mg/ml) containing either PKI (a and b) or STI (c and d) (both at 250 μM). After 60 min the cells were fixed and stained with TRITC-labeled anti-rabbit antibody. Fluorescence emanating from F'TCC is shown on the left (a and c) and from IgG on the right (b and d). Dually injected cells are indicated with arrows in panels a and c. Panels a and c correspond to panels b and d, respectively.](image-url)
FIG. 4. R'(R209K) alters the nuclear distribution of FITC-C. REF52 cells were injected with FITC-C (60 μM), incubated for 30 min, then re-injected with IgG (2 mg/ml)/R'(R209K) (120 μM) and fixed at 15 (a), 30 (c), or 60 min (e) after the second injection. Panels a, c, and e depict FITC fluorescence emanating from FITC-C and panels b, d, and f the same fields of cells stained for injected IgG/R'(R209K). Cells designated with arrows in panels a, c, and e received both FITC-C and IgG/R'(R209K) injections.

FIG. 5. Wild type R' is less effective than R'(R209K) in altering the nuclear distribution of C. Panel a shows the FITC fluorescence emanating from the FITC-C subunit and panel b the same field of cells stained for IgG co-injected with the R' subunit. The cells were fixed 60 min following cytoplasmic injection of R' (120 μM). Cells designated with arrows in panel a received both FITC-C and IgG/R'.

ence when sequential injections were performed with R'(R209K).

The ability of the wild type R' subunit to alter the nuclear distribution of C was also examined. In most dually injected cells, fluorescence emanating from the FITC-C subunit was evenly distributed throughout the nucleus and cytoplasm 60 min following the injection of wild type R' (Fig. 5, a and b). In a smaller number of cells (approximately 35%), FITC-C was localized primarily in the cytoplasm. Thus wild type R' was less effective than mutant R'(R209K) in depleting the nucleus of FITC-C. The slower rate of wild type R' compared with the mutant is most likely because it contains bound cAMP so that unlike R'(R209K), the dissociated R is more stable than the holoenzyme in the absence of active phosphodiesterases. However, PKIα was much more effective than either R subunit in depleting C from the nucleus, presumably because it can enter the nucleus itself and thus does not depend solely on diffusion of C out of the nucleus.

Nuclear Injection of the C Subunit—Because the subcellular distribution of the C subunit is highly dependent on its
aggregation state, it was important to better understand the translocation of the C subunit between the nucleus and the cytoplasm and to characterize more fully the role of its various inhibitors on the dynamics of the C subunit mobility. Having established that the free C subunit can readily enter the nucleus (3), the next question to resolve was whether the C subunit could also exit the nucleus. To answer this, the fluorescently labeled C subunit was microinjected directly into the nucleus. As a control, rabbit IgG, a protein that is known to be impermeable to the nuclear membrane (26, 27) was coinjected with FITC-C. After fixation, the cells were stained with a TRITC-conjugated anti-rabbit antibody to detect the coinjected IgG. Sixty min after injection, the distribution of the C subunit was identical whether the initial injection site was in the cytoplasm (Fig. 2a) or in the nucleus (Fig. 6b). In contrast, the IgG that was initially coinjected with FITC-C does not traverse the nuclear membrane (Fig. 6a).

**Nuclear Injection of Inhibitor Complexes R2C2 and PKI-C**—Upon holoenzyme dissociation, the C subunit equilibrates between the cytoplasm and the nucleus. However, upon lowering cAMP, it reassociates with the R subunit in the cytoplasm (2). Thus, we know that nuclear C can eventually return to the cytoplasm to form a holoenzyme complex. How it accomplishes this is less clear. To determine whether either of the inhibitors can influence nuclear export of C, both complexes were microinjected directly into the nucleus. A mixture containing FITC-C and a 2-fold molar excess of unlabeled recombinant PKI was prepared. Complex formation was assessed by the absence of enzyme activity. Previous experiments revealed that similar C-PKI complexes were stably maintained as assessed by the absence of enzyme activity in situ for at least 60 min following injection into rat fibroblasts (3). Preformed FITC-C-PKI complexes were introduced directly into the nucleus of REF52 cells and fluorescence distribution analyzed 60 min later. In contrast to the FITC-C subunit alone, injection of FITC-C-PKI complexes directly into the nucleus resulted in largely cytoplasmic fluorescence 60 min following injection (Fig. 6c).

Unlike either the C subunit alone or C-PKI complexes, C-R' holoenzyme introduced into the nucleus exhibited predominantly nuclear fluorescence (Fig. 6d). These results demonstrate that the C subunit reaches an equilibrium distribution between the nucleus and cytoplasm following nuclear injection but that PKI shifts this distribution toward the cytoplasm, while R' traps C and prevents its transit across the nuclear membrane. In the latter case, this is most likely a consequence of the large mass of holoenzyme (~176 kDa, Stokes’ radius of 47 Å), which prevents it from diffusing through nuclear pores (28, 29).

As a control to determine whether the formation of a stable complex between FITC-C and PKI is required for rapid exit from the nucleus, two experiments were done. In one case, FITC-C was coinjected with a 2-fold molar excess of a mutant form of PKI unable to bind C where Arg-18 and Arg-19 were both replaced with Ala. When coinjected with this mutant PKI, the distribution of C was identical to microinjection of FITC-C alone (Fig. 7, a and b). Likewise, when C was microinjected with a 60-fold excess of PKI(5-24), an inhibitor peptide derived from PKI, the C subunit was distributed between the

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**Fig. 6.** Nuclear injection of C-PKI complexes, in contrast to holoenzyme, prevents nuclear retention of the C subunit.

REF52 cells were injected in the nucleus with the components indicated and fixed 60 min later. a, FITC-C PKI (60 μM; 120 μM); rabbit IgG (2 mg/ml); b, FITC subunit (60 μM); c, FITC-C PKI complex (60 μM; 120 μM); d, holoenzyme consisting of the FITC-C subunit and R(R209K). Panel a shows fluorescence emanating from coinjected IgG detected by staining with a TRITC-conjugated anti-rabbit antibody. Panels b, c, and d show fluorescence emanating from the FITC-C subunit.
PKI Enhances the Nuclear Export of the C Subunit

FIG. 7. Neither PKI (R18A,R19A) nor PKI (5–24) alter the nuclear distribution of FTTC. REF52 cells were injected in the nucleus with (a and b) FTTC·PKI (R18A,R19A) (60 μM:120 μM) or with (c and d) FTTC·PKI (5–24) (60 μM:4 mM) and fixed 40 min after injection. Both mixtures also contained guinea pig IgG (4 mg/ml). After fixation, the cells were stained with a cascade blue-conjugated anti-guinea pig antibody. Fluorescence emanating from FTTC is shown in the left panels (a and c) and from IgG in the right panels (b and d).

FIG. 8. PKI enhances the rate at which C exits the nucleus. The time course of the nuclear to cytoplasmic intensity ratio is shown for individual cells injected in the nucleus with either (A) FTTC (60 μM) (t = 25.3 min, n = 2, 37 °C) or (B) FTTC·PKI (60 μM:120 μM) (t = 5.25 min, n = 5, 37 °C).

PKI Enhances Rate of C Subunit Exit from the Nucleus but Does Not Anchor the C Subunit in the Nucleus or Cytoplasm—

To examine the kinetics of C subunit exit from the nucleus following microinjection, quantitative low light level microscopy was used. FTTC alone or preformed FTTC·PKI complexes were injected into the nucleus. When injected as a complex with recombinant PKIα, the C subunit rapidly exited the nucleus (with a roughly exponential time course with time constant, t = 5.25 min) (Fig. 8B), while FTTC alone exited the nucleus at a slower rate (t = 17.1 min) (Fig. 8A). Regardless of the injection location (cytoplasm or nucleus), the final nuclear to cytoplasmic brightness ratios were 2.0 for injection of FTTC (indicating accumulation in the nucleus) and 0.6 for FTTC·PKI (indicating a cytoplasmic distribution). The rapid exit of complexed nuclear FTTC suggests an enhanced nuclear efflux rate. One alternative explanation was that complexation with PKI increased the mobility of the C subunit or decreased the binding of the C subunit to other components and that such mobilization occurred mainly in the nucleus rather than the cytoplasm. To test this hypothesis, fluorescence photobleaching recovery experiments were performed on the FTTC subunit microinjected with and without PKI. In the first set of experiments the labeled proteins were injected into the cytoplasm and a 30-μm diameter spot was bleached in the cytoplasm using high power 480-nm illumination. The time constants for fluorescence recovery were quite similar, 1.26 ± 0.44 min and 1.01 ± 0.40 min (n = 10) for cells injected with FTTC or FTTC·PKI complex, respectively (Fig. 9, A and B). Thus, there was no systematic difference in cytoplasmic mobility between FTTC and FTTC·PKI. The FTTC·PKI complex appeared to be quite mobile in the cytoplasm, because full fluorescence recovery was seen following photobleaching. Thus, it is unlikely that C·PKI localizes to the cytoplasm by binding to an immobile cytoplasmic protein. Because increased nuclear mobility of the C·PKI complex could also explain its more rapid exit from the nucleus, the mobility of both FTTC and FTTC·PKI complexes in the nucleus was examined in nuclear photobleaching recovery experiments. To allow the C·PKI complex to be maintained in the nucleus during photobleaching, the nuclear membrane was ruptured by intentionally overinjecting the nucleus. In control experiments, fluorescently labeled 70-kDa dextrans, which do not normally enter the nucleus, entered the nucleus following rupture of the membrane as shown in Fig. 9E. The FTTC·PKI complex also entered and was maintained in the nucleus following rupture of the nuclear membrane in a manner
PKI Enhances the Nuclear Export of the C Subunit

![Diagram]

**Fig. 9.** Photobleaching recovery shows that both PTKC and the PTKC-PKI complex are comparably mobile in both the cytoplasm and nucleus. Fluorescence recovery in the cytoplasm is shown for typical individual REF62 fibroblasts injected with either (A) PTKC or (B) PTKC-PKI. Rupture of the nuclear membrane (indicated by the arrow) permitted entry of both (E) 70-kDa β-dextran and (F) the PTKC-PKI complex. Typical nuclear photobleaching recovery traces are shown for cells injected with either (C) PTKC or (D) PTKC-PKI.

As shown in Fig. 9, C and D, there was little difference in the nuclear mobility of PTKC either alone or complexed with PKI. Thus, complexation with PKI accelerates the exit of C from the nucleus without affecting the lateral mobility of C in either the nucleus or cytoplasm. In addition, the fact that rupture of the nuclear membrane prevented the net export of PTKC-PKI argues for an active transport process that can be short-circuited, rather than a passive equilibrium binding preference for fixed components of the cytoplasm.

Both PKI and R(R209K) Block the C Subunit-mediated Changes in Gene Expression—Because phosphorylation of CREB in the nucleus is required for CRE-regulated gene expression and both PKI and R(R209K) restrict the C subunit to the cytoplasm, we tested their effects on gene expression mediated by the C subunit. Wistar rat thyroid cells stably transfected with a CRE-regulated β-galactosidase construct (30) were injected in the cytoplasm with PKI or R(R209K) (and rabbit IgG) and subsequently stimulated with 8-bromo-cAMP for 6 h. Following fixation, the cells were stained with the chromogenic substrate, 5-chloro-4-bromo-3-indolyl-β-d-galactopyranoside (X-gal) to monitor β-galactosidase expression and with an FITC-labeled anti-rabbit antibody to identify the injected cells. Injection of PKI and R(R209K) significantly reduced β-galactosidase expression compared with un.injected or control-injected cells (Table I). Thus, consistent with their ability to block catalytic activity and alter the intracellular distribution of the C subunit, both PKI and R(R209K) inhibited the induction of CRE-regulated promoter activity. Because the C subunit entry into the nucleus appears to be the rate-limiting step in CREB-mediated transcriptional activation, it is likely that PKI and R(R209K) inhibit CRE-regulated gene activity through inhibiting CREB phosphorylation (31).

**DISCUSSION**

Our results suggest a potential role for PKI in the regulation of the intracellular distribution of the catalytic subunit of cAMP-dependent protein kinase. Like the free C subunit, free PKI enters the nucleus following its injection into the cyto-
plasm of rat fibroblasts. Although detection of fluorescently labeled PKI required injection at high concentrations, preliminary experiments revealed that endogenous PKI can also be detected in the nucleus.² Two types of experiments revealed that PKI is capable of altering the intracellular distribution of the C subunit. First, nuclear injection of a C-PKI complex resulted in the accelerated exit of the C subunit from the nucleus compared with nuclear injection of C alone. In contrast, nuclear injection of the holoenzyme restricted C to the nucleus and prevented its localization in the cytoplasm. Second, in cells previously injected with the C subunit, a subsequent cytoplasmic injection of either PKI or type I R subunit resulted in the cytoplasmic localization of the C subunit, and PKI could deplete the nucleus of the C subunit much more rapidly than could R. This temporal difference is likely due to the fact that PKI and R alter the distribution of the C subunit by different mechanisms. PKI enters the nucleus where it can bind C and speed its export from the nucleus. In contrast, the R subunit does not traverse the nuclear membrane and therefore can only trap C as it shuttles between cellular compartments. There are reports describing a nuclear reservoir of endogenous R subunits as determined by indirect immunofluorescence (32–35). Our studies have all used the R(α) subunit although in our hands R(α) behaves similarly.² An excess of PKI relative to the C subunit was used in these experiments to allow for PKI binding to any endogenous free C subunit in the injected cells. Hence, investigation of the activity of endogenous PKI, R and C will be necessary to test the physiological relevance of these relationships.

Based upon these results, we propose a model in which PKI works in conjunction with the R subunit to regulate both the activity and subcellular localization of the C subunit. Under basal conditions, C is cytoplasmic because it exists as holoenzyme, which is too large to cross the nuclear membrane (1, 2). Endogenous holoenzyme is also cytoplasmic as determined by immunostaining with an antibody raised to Co (not shown). Treating cells with an agonist that increases cAMP levels causes dissociation of C and R. In the absence of PKI, the C subunit can move freely between the cytoplasm and the nucleus. At equilibrium, C is found in both the cytoplasm and the nucleus following injection into either compartment. When PKI is present, it can rapidly enter the nucleus, bind C, and return it to the cytoplasm. PKI-assisted exit of C from the nucleus is much faster than exit of C by itself. In this model, the main effect of constitutively expressed PKI may be to set a threshold so that low levels of cAMP cause release of some free C in the cytoplasm but rather little accumulation in the nucleus as long as free PKI is available to shuttle C back out. Only at higher levels of cAMP, where the C subunits are released in excess of the PKI reserve, would nuclear accumulation of C build up significantly. When cAMP levels decrease, R will be depleted of cAMP and cannot only bind any free cytoplasmic C but can also wrest C from cytoplasmic C-PKI complexes, because at low cAMP levels, C binds more tightly to R than to PKI.³ It is not yet clear whether PKI accompanies C and accumulates in the cytoplasm because, under current labeling conditions, detection of PKI required injection at high concentrations. In addition, we cannot exclude that the C-PKI complex may have additional destinations, including degradative pathways. Nevertheless, PKI has the potential to limit the access of C to the nucleus and assist, directly or indirectly, in the cytoplasmic reassociation of C with R.

If PKI functions physiologically to help export C from the nucleus, then its expression should be regulated. If expressed constitutively and at sufficient levels, it could reduce or block cAMP-mediated gene expression, because, in the absence of free C, PKI would be localized in the nucleus. PKI would thus selectively block the nuclear effects of cAMP activation, for example, phosphorylation of CREB (8). Up-regulation of PKI would further prevent cAMP-mediated gene expression. Down-regulation of PKI would not only enhance the overall activity of C but would also selectively enhance the nuclear effects of cAMP by allowing more C to access the nucleus. Where it has been examined, cellular levels of PKI are lower than those of C (11). Maximal estimates suggest that there is only enough inhibitor to block 20% of the C subunit in skeletal muscle (36). This, of course, represents an average value and does not consider PKI levels in individual cells. Nevertheless, the lower abundance of PKI is not inconsistent with its putative role in nuclear surveillance where the amount of the C subunit which enters the nucleus would be less than the total cellular content of holoenzyme. Moreover, if PKI functions to return C to the cytoplasm where it reassociates with R, then PKI can recycle to the nucleus. In vitro, R will compete PKI from a C-PKI complex,³ suggesting that binding of PKI to C is not irreversible. There is little evidence so far to support differential regulation of PKI levels (reviewed in Ref. 11). One of the few examples is the effect of vitamin D in the kidney. In this tissue, vitamin D is known to down-regulate the expression of PKI (37).

Recent evidence suggests that C cycles between the nucleus and cytoplasm by passive bi-directional diffusion through the nuclear pore.⁴ For example, the rates at which C enters and exits the nucleus are consistent with simple diffusion (Fig. 7). Although C equilibrates throughout the cell, injected C-PKI complexes were previously shown to be cytoplasmic (3) even though this complex is still small enough to diffuse through the nuclear pore (28, 29), 26.1 Å for C versus 31Å for the C-

Table I

| Injected material* | Number of injected cells analyzed | β-Galactosidase expression in injected cells | Number of un.injected cells analyzed | β-Galactosidase expression in un injected cells |
|-------------------|----------------------------------|-------------------------------------------|-------------------------------------|---------------------------------------------|
| PKI               | 439                              | 9.0 (95% CI = 6–12)²                      | 1181                                | 85 (95% CI = 83–87)                         |
| R(α) (R209K)      | 418                              | 7.0 (95% CI = 6–9)                        | 574                                 | 75 (95% CI = 71–79)                         |
| Rabbit IgG        | 465                              | 85 (95% CI = 82–88)                       | 898                                 | 85 (95% CI = 83–87)                         |

* PKI (250 μm) and R(α) (R209K) (120 μM) were coinjected with rabbit IgG (4 mg/ml) to identify the injected cells.

WRT CRE cells (30) were injected and then stimulated with 1 mM 8BrcAMP for 6 h. Following histochemical staining to monitor β-galactosidase expression, the cells were stained with FITC anti-rabbit IgG to identify the injected cells. Injected cells were scored for the presence (blue) or absence (white) of β-galactosidase expression.

² 90% confidence intervals were calculated using the standard error of proportion, Sp.

² D. A. Fantozzi, W. Wen, S. S. Taylor, and J. L. Meinloth, unpublished observations.
PKI complex (38). Thus it was possible that C entered the nucleus by active transport and that both PKI and R masked a nuclear translocation signal (3). Indeed, a putative translocation signal is found on the surface of C (residues 189–192, Lys-Arg-Val-Lys), and this site may be masked by PKI and R based on the crystal structure of the C subunit complexed with an inhibitor peptide from PKI (39–41). Mutation of this site, however, did not prevent nuclear accumulation of C. Indeed, all of our results to date are consistent with a passive bi-directional model for C subunit entry into the nucleus; there is no demonstrated requirement for a nuclear localization signal. However, neither the masking of a nuclear import signal on C by PKI or the simple passive diffusion of C would explain why C-PKI complexes leave the nucleus faster than C alone enters or leaves it. There are several potential explanations for the rapid exit of C from the nucleus following nuclear injection of C-PKI complexes. First, it was formally possible that enzymatic activity was required for retention of C subunit within the nucleus. However, we showed previously that the enzymatically inactive C subunit, generated either by treatment of the C subunit with N-ethylmaleimide or by complexation with an inhibitory peptide generated from PKI, did not prevent nuclear accumulation of C. This finding indicated that enzymatic activity was not required for retention of C within the nucleus. However, we showed previously that the enzymatically inactive C subunit, generated either by treatment of the C subunit with N-ethylmaleimide or by complexation with an inhibitory peptide generated from PKI, did not prevent nuclear accumulation of C. Third, it was possible that PKI acted to displace C from a nuclear binding site. However, lateral diffusion of C was not affected by PKI in either the nucleus or cytoplasm. In the presence of PKI, the C subunit enters the nucleus by diffusion and is actively exported from the nucleus by PKI.

The nature of the putative nuclear export signal remains to be identified. The signal might reside on PKI but be masked by diffusion. In the presence of PKI, the C subunit enters and exits the nucleus by passive binuclear diffusion. In the presence of PKI, the C subunit enters the nucleus by diffusion and is actively exported from the nucleus by PKI.

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PKI Enhances the Nuclear Export of the C Subunit

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