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Movement of the Free Catalytic Subunit of cAMP-dependent Protein Kinase into and out of the Nucleus Can Be Explained by Diffusion

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The catalytic (C) subunit of cyclic AMP (cAMP) dependent protein kinase (PKA) has previously been shown to enter and exit the nucleus of cells when intracellular cAMP is raised and lowered, respectively. To determine the mechanism of nuclear translocation, fluorescently labeled C subunit was injected into living REF52 fibroblasts either as free C subunit or in the form of holoenzyme (PKA) in which the catalytic and regulatory subunits were labeled with fluorescein and rhodamine, respectively. Quantification of nuclear and cytoplasmic fluorescence intensities revealed that free C subunit nuclear accumulation was most similar to that of macromolecules that diffuse into the nucleus. A glutathione S-transferase-C subunit fusion protein did not enter the nucleus following cytoplasmic microinjection. Puncturing the nuclear membrane did not decrease the nuclear concentration of C subunit, and C subunit entry into the nucleus did not appear to be saturable. Cooling or depleting cells of energy failed to block movement of C subunit into the nucleus. Photobleaching experiments showed that even after reaching equilibrium at high [cAMP], individual molecules of C subunit continued to leave the nucleus at approximately the same rate that they had originally entered. These results indicate that diffusion is sufficient to explain most aspects of C subunit subcellular localization.

INTRODUCTION

How signals are transmitted from the plasma membrane to the nucleus is a subject of fundamental interest in many biological processes. In pathways involving the second messenger, cyclic AMP (cAMP), receptor occupation leads to activation of adenylate cyclase which in turn produces an increase in intracellular [cAMP]. cAMP binds to the regulatory (R) subunits of cAMP dependent protein kinase (PKA), which is a tetramer consisting of two R and two catalytic (C) subunits. Upon binding cAMP the holoenzyme dissociates into an R dimer and two active C subunits. The free C subunits phosphorylate protein targets in both the cytoplasm and nucleus. Nuclear phosphorylation targets include members of the CREB/ATF family of transcription activators, which, when phosphorylated by C subunit, can induce transcription in genes containing cAMP response elements (CREs) (reviewed in Brindle and Montminy, 1992).

The subcellular localization of PKA subunits has been the subject of debate (reviewed in Lohmann and Walter, 1984). Recent studies using either fluorescently labeled C subunit (Meinkoth *et al.*, 1990; Adams *et al.*, 1991) or immunocytochemistry (Kuettel *et al.*, 1985; Nigg *et al.*, 1985) reveal that both the C and R subunits remain in the cytoplasm when cAMP is low, because holoenzyme is too large to enter the nucleus. When cAMP is increased, the active C subunit monomers dissociate from the R dimer and are able to translocate into the nucleus. Upon subsequent cAMP decrease, the C subunit exits the nucleus (Nigg *et al.*, 1985; Adams *et al.*, 1991). The cytoplasmic localization of the R subunit, unlike the C subunit, appears to be independent of cAMP fluctuation. The molecular mechanism responsible for movement of the C subunit between the cytoplasm and nucleus is not known. Free C subunit could enter the nucleus either by active uptake, passive diffusion, or by an as yet undefined mechanism based either on cAMP or phosphorylation. The C subunit con-

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tains many basic lysines and arginines that could be the basis for a nuclear localization signal (NLS). Upon injection into the cell, free C subunit appears to concentrate in the nucleus of cells but not when injected in the presence of excess heat stable protein kinase inhibitor (PKI) (Fantozzi *et al.*, 1992). Thus PKI binding to C subunit may mask a NLS on the C subunit and block translocation. Previous evidence in favor of nuclear translocation via passive diffusion is based mainly on the size of C subunit (≈41 kDa, 27 Å), which is smaller than the radius of the nuclear pore (45–55 Å). The fact that the C subunit can exit the nucleus also provides evidence in favor of passive diffusion as the mechanism for nuclear translocation because nucleoplasmin, a protein containing a NLS, fails to exit the nucleus when directly microinjected into the nucleus (Schultz and Peters, 1987). Once within the nucleus, free C subunit either remains sequestered there or diffuses between the nucleus and cytoplasm. In addition, the molecular mechanism by which C subunit exits the nucleus when [cAMP] decreases is as yet undefined, although the final step involved in cytoplasmic localization at low [cAMP] is most likely binding of the C subunit to R subunits restricted to the cytoplasm.

To quantitatively assess how the C subunit both localized and translocated between the cytoplasm and nucleus, experiments using fluorescently labeled C subunit were performed. Fluorescently labeled C subunit was injected into living REF52 fibroblasts either as free fluorescein-labeled C subunit or in the form of holoenzyme (PKA) in which the C and R subunits were labeled with fluorescein and rhodamine, respectively. In most experiments using doubly labeled holoenzyme (fluorescein-labeled catalytic rhodamine-labeled regulatory [FlCRhR]), the degree of kinase activation was assessed by monitoring fluorescence resonance energy transfer (FRET) between the R and C subunits (Adams *et al.*, 1991). Nuclear accumulation of C subunit was quantitatively compared to that of other relatively low molecular weight macromolecules reported to enter the nucleus either by diffusion (10 kDa dextran or soybean trypsin inhibitor [STI]) or active uptake (histone H1 or human serum albumin coupled to SV40 T-antigen signal sequences [ss-HSA]). Experiments were also performed attempting to block active transport of the C subunit into the nucleus, such as lowering the temperature to 9°C and treating cells with metabolic inhibitors or competitive substrates (excess histone H1). Because active import of proteins into the nucleus has been shown to be a saturable process, we also investigated whether nuclear import of C subunit was saturable. Finally, quantitative measurements of free C subunit movement between the nucleus and cytoplasm were made at high effective [cAMP]. In these experiments, the cytoplasmic C subunit was photobleached, and redistribution of the fluorescent nuclear protein was observed. Although the experiments in this paper focus primarily on the specific question of how the C subunit enters and exits the nucleus, the techniques used could be extended to elucidate the mechanism by which any protein moves into and out of the nucleus.

MATERIALS AND METHODS

Cell Culture and Microinjection

REF52 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) calf serum, 50 units/ml penicillin, and 50 μM streptomycin. Cells were seeded on thickness no. 1 glass coverslips ≥24 h before use in experiments. Fluorescently labeled protein was introduced by microinjection with glass needles filled with either 7–40 μM FlCRhR or 4–500 μM C subunit as described previously (Adams *et al.*, 1991). The resulting intracellular protein concentrations were 70 nM to 4 μM (0.013–0.72 mg/ml) for FlCRhR and 40 nM to 50 μM (0.0016–2.0 mg/ml) for C subunit assuming an injection volume of 1–10% of the cell volume. The fluorescence intensities of C subunit within cells were verified to span a range of a 1000-fold. All cells were injected into the cytoplasm. In some experiments the microinjection pipettes were used intentionally to puncture the nuclear membrane.

Fluorescent Labeling of Proteins

Doubly labeled PKA (FlCRhR) was prepared as described (Adams *et al.*, 1991) with the exception that 1 mg/ml recombinant R_α^{II} (Scott *et al.*, 1990) labeled with 100 μM 5'(6')-carboxytetramethylrhodamine succinimidyl ester (Molecular Probes, Eugene, OR) was used instead of R_α^{I} labeled with tetramethylrhodamine isothiocyanate (TRITC) (Adams *et al.*, 1993; Bacskai *et al.*, 1993). Histone H1 and STI (Sigma, St. Louis, MO) were labeled with fluorescein isothiocyanate (FITC) in a bicarbonate buffer as described (Breeuwer and Goldfarb, 1990), with the exception that FITC from Molecular Probes was utilized instead of FITC on Celite from Sigma. ss-HSA labeled with rhodamine isothiocyanate was a gift of Dr. Douglass Forbes (University of California San Diego). Fluorescein-labeled dextrans were from Molecular Probes. Proteins were separated on 3-ml columns of Sephadex G-25 pre-equilibrated with 25 mM potassium phosphate pH 6.8, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 5% glycerol and then dialyzed against the same buffer overnight at 4°C. The protein was concentrated in injection buffer (25 mM potassium phosphate pH 7.3, 1 mM EDTA, 0.5 mM 2-mercaptoethanol, and 2.5% glycerol) using Centricon (Amicon, Beverly, MA) concentrators.

Preparation of Glutathione S-Transferase (GST)-C Fusion Protein

The cDNA encoding the mouse C_α subunit (Slice and Taylor, 1989) was subcloned into the *Nco* I/*Hin*dIII sites of the pGEX-2T vector of Guan and Dixon (1991). The GST-C subunit fusion protein was expressed in *E. coli* and purified by affinity chromatography on a glutathione-agarose column as previously described (Guan and Dixon, 1991). The purified fusion protein was ≥95% pure based on Western blot analysis with a C subunit antibody. Antibody was prepared against recombinant mouse C_α subunit and then purified by affinity chromatography on a C-subunit-agarose column. The activity of the fusion protein was performed using the spectrophotometric method described by Cook *et al.* (1982).

Fluorescence Imaging and Photobleaching

Three different imaging systems were used. Conventional inverted fluorescence microscopes, Zeiss IM35 or Axiovert (Thornwood, NY) coupled to a silicon-intensified target (Dage-MTI, Michigan City, IN) or cooled charge-coupled-device camera (Photometrics, Tucson, AZ), respectively, were used to track movement of protein from cytoplasm

to nucleus. The 12-bit dynamic range and variable integration time of the charge-coupled-device camera were particularly useful to quantify the very wide range ($\sim 10^3$) of fluorescence intensities and protein concentrations. To monitor movement in the opposite direction, it was necessary to create an apparent excess of fluorescent protein in the nucleus and watch the evolution of this initial state. We considered injecting the fluorescent protein directly into the nucleus, but this approach often suffered from delayed or incomplete resealing of the nuclear membrane. Instead, an indirect approach using selective photobleaching of the cytoplasm was more reliable. Cells were injected into the cytoplasm, and the protein was allowed to equilibrate while observing with a custom-made confocal microscope (Tsien, 1990; Bacskai *et al.*, 1993). A slightly oversized mask was placed in a secondary image plane of the microscope to shield the nucleus from photobleaching. The 488-nm laser power was increased by about 50-fold for photobleaching; typical bleaching times were 15–60 s of continuous scanning resulting in about half of the cytoplasmic labeled protein being bleached. The laser power was then reduced, and the mask was removed during intermittent imaging of the subsequent redistribution. This method had the further advantage that both nuclear entry and exit could be sequentially monitored in the same cell with a single cytoplasmic injection. Photobleaching of fluorescently labeled protein in the nucleus has been used previously (reviewed by Peters, 1986) to measure transport rates from the cytoplasm to the nucleus.

Energy transfer was measured by monitoring the ratio of fluorescence intensities at fluorescein (500–530 nm) and rhodamine (>570 nm) emission wavelengths when illuminated at fluorescein excitation wavelengths (485 or 488 nm). Data from images was analyzed by drawing a circular spot over the area of interest and averaging the intensity as described previously (Tsien and Harootunian, 1990). Two or more cytoplasmic spots were used in most experiments to control for artifacts such as cell movement or redistribution of protein within the cytoplasm. Because both fluorescein and rhodamine emission intensities were collected in most experiments, both nuclear to cytoplasmic intensity ratios and fluorescein to rhodamine emission intensity ratios were determined.

RESULTS

Final Nuclear to Cytoplasmic Concentration Ratios of the C Subunit of PKA Resemble Those of Proteins or Macromolecules that Enter the Nucleus by Diffusion

To determine whether the C subunit of PKA enters the nucleus of cells by passive diffusion or by active uptake, several experiments were performed. Final nuclear to cytoplasmic fluorescence intensity ratios of the C subunit were quantitatively compared to those of control fluorescently labeled macromolecules that enter the nucleus by known mechanisms. Dextran (70 kDa) was chosen as the control large macromolecule that does not pass through the nuclear pore. STI (21 kDa) and dextran (10 kDa) were chosen as control macromolecules that enter the nucleus via diffusion (Peters, 1986; Dabauvalle *et al.*, 1988; Breeuwer and Goldfarb, 1990). Finally, histone H1 (21 kDa) and ss-HSA were chosen as examples of proteins that enter the nucleus by active transport (Breeuwer and Goldfarb, 1990; Forbes, 1992). After injection into REF52 fibroblasts, fluorescently labeled holoenzyme (FlCRhR) remained in the cytoplasm. When cells were treated with forskolin or prostaglandin E_1 (PGE_1), cAMP increased causing C subunits to rapidly

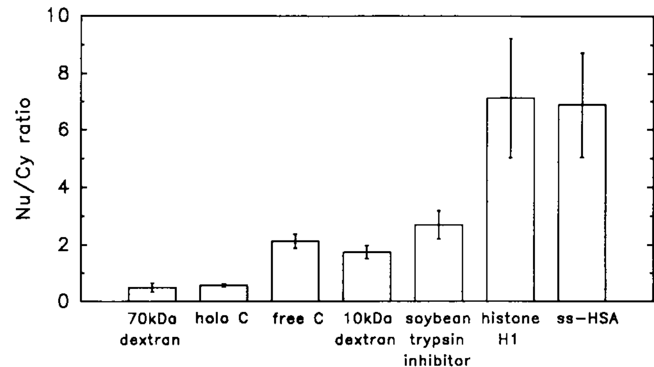


Figure 1. Nuclear to cytoplasmic fluorescence brightness ratios are plotted for fluorescently labeled 70-kDa dextran, PKA holoenzyme, free C subunit, 10-kDa dextran, STI inhibitor, histone H1, and ss-HSA. All cells were injected in the cytoplasm and observed 30 min after injection.

separate from the R subunit and gradually enter the nucleus. The final nuclear to cytoplasmic fluorescence intensity ratio of free C subunit (Figure 1), although greater than one, most closely resembled those of 10-kDa fluorescein dextran and STI, two macromolecules that enter the nucleus by diffusion. The final nuclear to cytoplasmic brightness ratio of free C subunit was markedly less than that of both histone H1 and ss-HSA. Perinuclear localization of the C subunit was not evident in any of the experiments performed. Thus, quantitative analysis of protein concentrations appears consistent with diffusion of C subunit into the nucleus.

In contrast to free C subunit, the nuclear to cytoplasmic ratio of C subunit injected in the form of holoenzyme closely resembled that of 70-kDa dextran (Figure 1). The large size of 70-kDa dextran (>55Å, reviewed by Peters, 1986) prevents its passage through nuclear pores. Nuclear fluorescence is not zero because of optical contamination from the cytoplasm above or below the nucleus. An attempt to optically section the cell by reducing the size of the pinhole on the confocal microscope decreased but did not bring to zero the nuclear to cytoplasmic intensity ratio of 70-kDa dextran. Thus, PKA holoenzyme distribution is similar to large macromolecules that are excluded from the nucleus based on size, whereas free C subunits localize in the nucleus to an extent consistent with other macromolecules known to enter the nucleus by passive diffusion.

A GST-C Fusion Protein Does Not Enter the Nucleus

To further examine whether C subunit enters the nucleus by active transport or passive diffusion, a GST-C subunit fusion protein was used because this molecule should be too large to enter the nucleus by passive diffusion. Coomassie-stained sodium dodecyl sulfate (SDS) polyacrylamide gels of purified C subunit and the GST-C fusion protein are shown in Figure 2A, lanes 1 and

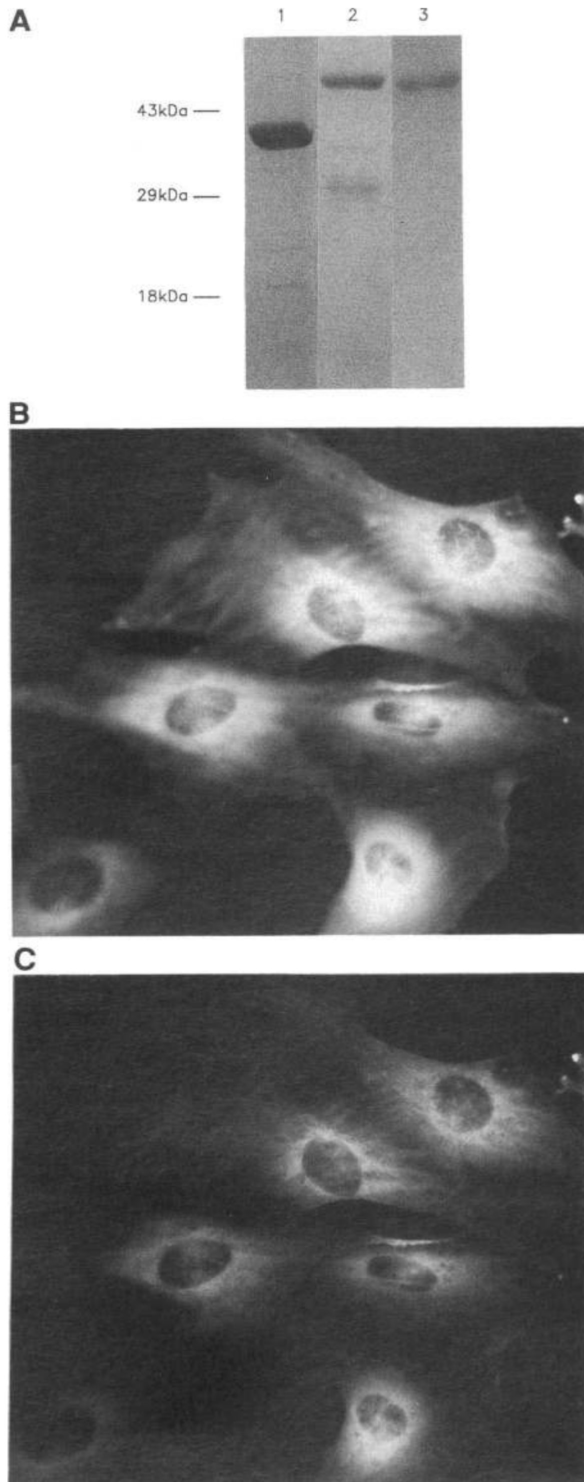


Figure 2. GST-C is retained in the cytoplasm. (A) SDS-polyacrylamide gel electrophoresis of the C subunit and GST-C fusion protein stained with Coomassie Blue (lanes 1 and 2) and a Western blot of GST-C (lane 3) using a rabbit polyclonal anti-C antibody. (B and C) REF52 cells were injected into the cytoplasm with a pipette containing GST-C (16 μM) and guinea pig IgG (4 mg/ml), incubated for 60 min, and fixed and stained with the same anti-C antibody, a rhodamine-conjugated anti-rabbit antibody and an AMCA-conjugated anti-guinea pig antibody. B illustrates cells stained for the presence of GST-C, and C shows the same field of cells stained for coinjected IgG. The cells were observed using a Zeiss Axiophot fluorescence microscope and photographed using Kodak TMY film.

2, respectively. Western blotting demonstrated that the GST-C fusion protein was recognized by a polyclonal antibody raised to C (Figure 2A, lane 3). The GST-C fusion protein exhibited enzymatic activity similar to recombinant C subunit, and that activity was inhibited by both type I R subunit and PKI. Because the only sequence resembling a nuclear localization signal on C (KRVK, 189–192) is on the surface of C that binds PKI and the R^I regulatory subunit of PKA (Gibbs *et al.*, 1992), this region must be exposed on the GST-C fusion protein. The GST-C fusion protein was injected into the cytoplasm of REF52 cells, and its distribution was assessed by indirect immunofluorescence using an antibody against the C subunit. Guinea pig IgG was coinjected with GST-C, and its distribution was also assessed. Both GST-C and guinea pig IgG were restricted to the cytoplasm 60 min after injection as shown in Figure 2, B and C. This is in stark contrast to wild-type C subunit, which accumulates in the nucleus within 30 min of injection into the cytoplasm.

Rupture of the Nuclear Membrane Does Not Alter Intracellular Distribution of Free C Subunit

To directly test the contribution of active unidirectional transport across the nuclear membrane to the intracellular distribution of C subunit, the nuclear membrane was deliberately broken by the micropipette, and subsequent C subunit intracellular redistribution was observed. As shown in Figure 3A, disruption of the nuclear membrane does not markedly alter the relative concentration of C subunit in the nucleus versus the cytoplasm. By contrast, ss-HSA, which enters the nucleus by active import, rapidly exited the nucleus after rupture of the nuclear membrane as shown in Figure 3B. These results provide further evidence against active unidirectional nuclear import of free C subunit, because short circuiting the nuclear membrane did not markedly alter the subcellular distribution of C subunit but did radically decrease the nuclear concentration of ss-HSA.

Movement of C Subunit into the Nucleus Is Not Saturable

Another approach to selectively block active uptake of proteins into the nucleus is to use a competitive inhibitor of active nuclear protein import. Active import of proteins into the nucleus has been shown to be a saturable process (Goldfarb *et al.*, 1986) and should thus be sensitive to competitive inhibition by other substrates such as histone H1. To avoid aggregation when histone H1 and FlCRhR were mixed at high concentration in the

injection pipette, cells were first injected with FlCRhR and subsequently injected with a 1 mM pipette concentration of histone H1. Treating cells with 25 μM forskolin elicited an increase in [cAMP] and dissociation of FlCRhR. The subsequent nuclear uptake of free C subunit was not altered by injection of histone H1 (Figure 4A), suggesting that nuclear import of C subunit was not saturable. The time course for C subunit movement into the nucleus shown in Figure 4A was well within the norm for control cells not injected with histone H1 (τ = 28 ± 13 min, n = 10). Active nuclear import of ss-HSA was effectively blocked by preinjecting cells with histone H1 (n = 4) as shown in Figure 4B.

We next directly checked for any dependence of nuclear entry rate and steady-state nuclear/cytoplasmic ratio on the concentration of C subunit itself. If nuclear entry of C subunit were a saturable process, apparent time constants should increase with increasing protein concentration. If C subunit could enter the nucleus both by a unidirectional saturable mechanism and by nonsaturable diffusion, as has been reported for histone H1 (Breeuwer and Goldfarb, 1990), then steady-state nuclear/cytoplasmic ratios should decrease with increasing C subunit concentrations and consequent saturation of the import mechanism. If C subunit were binding to a

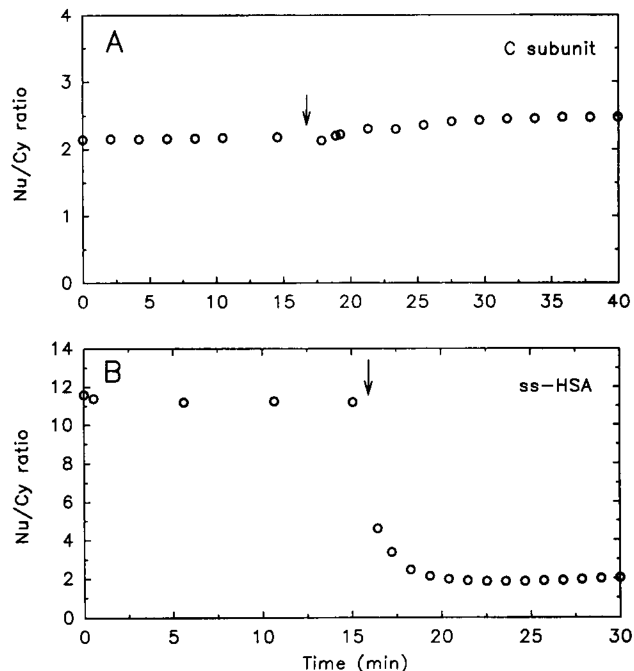


Figure 3. Puncturing the nuclear membrane does not alter the distribution of C subunit. Cells were injected in the cytoplasm with C subunit (A) or ss-HSA. After the C subunit or ss-HSA had equilibrated between cytoplasm and nucleus, the nuclear membrane was punctured at the time indicated by the arrow. Rupture of the nuclear membrane did not markedly alter the distribution of C subunit (A) but drastically reduced the concentration of ss-HSA (B) in the nucleus.

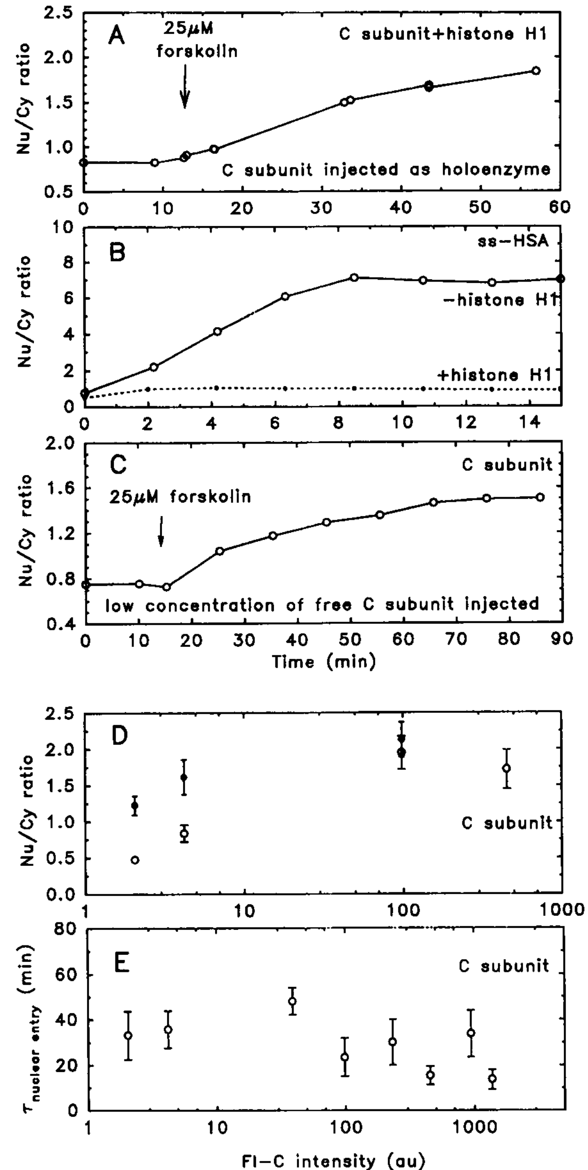


Figure 4. Entry of C subunit into the nucleus is not saturable. (A) C subunit enters the nucleus of cells injected with histone H1. The nuclear to cytoplasmic brightness ratio of FITC-labeled C subunit gradually increases after kinase activation. The REF52 fibroblast was initially injected with FlCRhR and subsequently injected before kinase activation via a pipette containing 800 μM histone H1. (B) ss-HSA entry into the nucleus is blocked by injecting cells with histone H1 (●) shortly before injection of ss-HSA. The time course for nuclear entry of ss-HSA without histone H1 preinjection (○) is also shown. (C) When injected at low concentrations (50 nM–1 μM final intracellular concentration, ≈300 nM for this cell), C subunit remains largely cytoplasmic until [cAMP] is increased by treating cells with forskolin. (D) The nuclear to cytoplasmic intensity ratio of C subunit is plotted as a function of the average intensity of C subunit in the cell. ●, data taken at high [cAMP]; ●, data taken at low [cAMP]. The lowest data point in D may be a slight underestimate of the true Nu/Cy ratio because of autofluorescence in the cytoplasm. (E) The time constant for entry of the mobile fraction of C subunit into the nucleus is plotted as a function of the intensity of injected C subunit. The data at the two lowest concentrations of C were obtained in the presence of high [cAMP] to reduce cytoplasmic immobilization of C.

limited number of saturable sites in either nucleus or cytoplasm, steady-state nuclear/cytoplasmic ratios should decrease or increase respectively as C subunit concentrations were increased beyond the binding capacity of either compartment. C subunit concentrations were varied from about 40 nM to 50 μM as described in MATERIALS AND METHODS. Endogenous concentrations of C subunit are not precisely known for these fibroblasts but are between 0.3 and 1 μM in a variety of cell types (Hofmann *et al.*, 1977; Lohmann and Walter, 1984; Hagiwara *et al.*, 1993). Figure 4C shows that at ~0.3 μM, an order of magnitude below that used in the previous figures, the C subunit tended to remain predominantly cytoplasmic until [cAMP] was elevated. Therefore nuclear/cytoplasmic ratios increased with increasing C subunit concentrations (Figure 4D, ○), though the slope was much reduced by high [cAMP] (●). These results suggest that the dominant binding sites for exogenous C subunit are cytoplasmic rather than nuclear, have a capacity on the order of 1 μM, and are either endogenous R subunits or closely related proteins because they release C upon elevation of [cAMP]. No evidence could be found either for unidirectional import becoming dominant at low C subunit concentrations or for saturable nuclear binding sites. C subunit nuclear import did not seem self-saturable, because the time constant for nuclear entry of the mobile fraction of C subunit (Figure 4E) if anything decreased slightly as protein concentration was increased by three orders of magnitude. Thus, both the histone H1 coinjection data and the effects of varying the C subunit concentration argue against saturable transport processes as the primary means by which C subunit enters the nucleus.

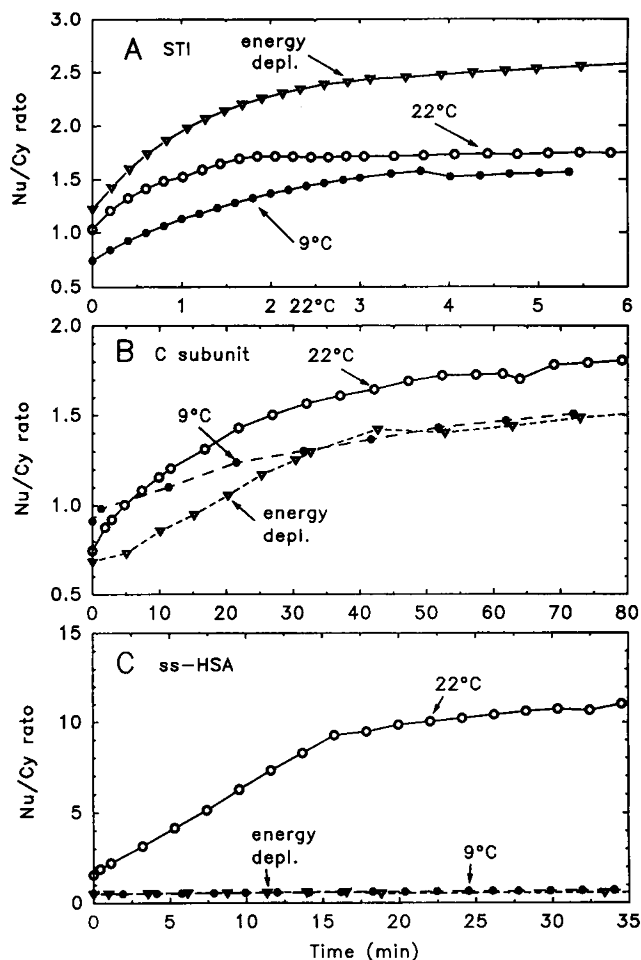


Figure 5. Cooling or energy depletion does not block nuclear import of C subunit. Typical time courses of nuclear entry of STI (A), C subunit (B), or ss-HSA (C) are shown for individual cells at 22°C (○), 9° (●), or at 22°C after treatment for 30 min with 10 mM sodium azide and 6 mM deoxyglucose to deplete ATP (▽).

Cooling and Energy Depletion Do not Block Nuclear Import of C Subunit but Do Block Nuclear Import of ss-HSA

To further investigate the mechanism by which C subunit entered the nucleus, we utilized protocols that specifically inhibited active transport of proteins into the nucleus. Although ATP-dependent active uptake of proteins is blocked by cooling cells (Schultz and Peters, 1987; Richardson *et al.*, 1988), diffusion of 10-kDa dextran into the nucleus is not affected by temperature (Schultz and Peters, 1987). Energy depletion has also been shown to selectively block active import of protein into the nucleus (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988). We verified that nuclear entry of STI, which enters the nucleus by diffusion, was slowed by a factor of three (τ = 0.62 min to τ = 1.91 min) when cells were cooled from 22°C to 9°C (Figure 5A). C subunit nuclear entry was likewise slowed by the same factor of threefold (τ = 28.0 min to τ = 90.2 min) by the same temperature drop, as shown in Figure 5B. C subunit entered the nucleus at 9°C regardless of the concentration of protein injected provided [cAMP] was high. By contrast the nuclear entry rate of ss-HSA was drastically slowed by at least a factor of 15 when cells were cooled from 22°C to 9°C (τ = 17.4 min to τ $\geq$ 268 min) as shown in Figure 5C.

Next, cells were depleted of ATP by incubation in 5.6 mM deoxyglucose and 10 mM sodium azide for 30 min before injection. Because decreasing the level of ATP in cells might also decrease the level of cAMP, low levels of C subunit were avoided in these experiments. Nuclear entry rates of STI and C subunit (40 μM in the injection pipette) were slowed by 2.4-fold to τ = 1.49 and τ = 67.9 min, respectively, following energy depletion (Figure 5, A and B). By contrast, energy depletion completely blocked nuclear entry of ss-HSA (Figure 5C). In addition, treatment with azide and deoxyglucose after allowing C subunit to equilibrate between the nucleus and cytoplasm failed to reduce the amount of C subunit

in the nucleus providing further evidence against ATP-dependent transport as the mechanism by which C subunit enters the nucleus. The slight decrease in relative amount of C subunit in the nucleus can most likely be traced to a reduction because of energy depletion (from >80% to 50%) in the fraction of mobile C subunit in the cell. Thus, cooling and energy depletion, which block active import of proteins into the nucleus, only slightly retarded the entry of both STI and C subunit into the nucleus but dramatically blocked nuclear import of ss-HSA.

Free C Subunit Exits the Nucleus

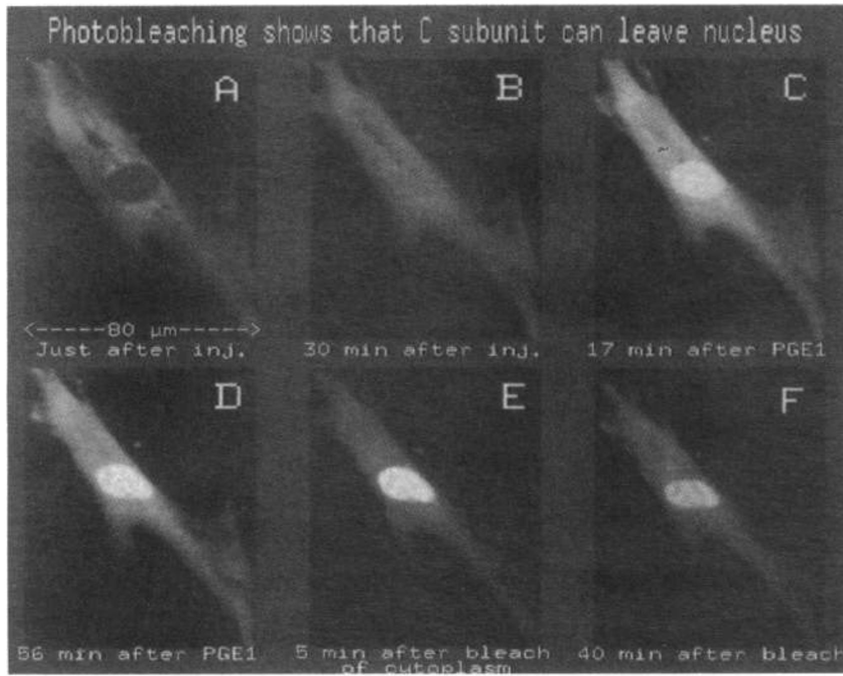
To determine whether the C subunit was continually shuttling between the nucleus and cytoplasm or sequestered in the nucleus, we devised a set of experiments using fluorescence photobleaching recovery to monitor protein movement at equilibrium (Figure 6). After cytoplasmic microinjection of FlCRhR, the nuclear to cytoplasmic fluorescence intensity ratio increased slightly because of a small amount of contaminating free C subunit in this specific batch of FlCRhR. After allowing the nuclear to cytoplasmic intensity ratio to equilibrate, [cAMP] was increased by treating cells with 10 μM PGE_1. PKA holoenzyme dissociated as measured by energy transfer, and the C subunit entered the nucleus as measured by an increase in the nuclear to cytoplasmic fluorescence intensity ratios. After the nuclear to cytoplasmic intensity ratio had reached equilibrium, the cytoplasmic protein was bleached to 50% of the original intensity. As a result of photobleaching, there was an instant qualitative (Figure 6, A–F) and quantitative (Figure 6G) increase in the nuclear to cytoplasmic brightness ratio, followed by a gradual decrease over the course of the next hour. Any changes in cell shape (Figure 6, A–F) were most likely because of cell rounding as a result of free and active C perturbing the cytoskeleton of the REF52 fibroblasts (Lamb *et al.*, 1988, 1989; Meinkoth *et al.*, 1990). In most experiments the N/C ratio returned to the same level as before photobleaching, although on occasion a fraction of protein appeared to be immobile. This result implies that the unbleached free C subunit of PKA can exit from the nucleus even in the presence of high [cAMP].

Cytoplasmic photobleaching experiments were also performed on cells injected with FITC-labeled free C subunit at low [cAMP]. Once again, photobleaching was delayed until the microinjected C subunit had equilibrated between the cytoplasm and nucleus. After photobleaching (Figure 6H), the C subunit could be seen exiting the nucleus with a time course (τ = 22.9 min) indistinguishable to that of free C subunit at high [cAMP]. The average exit and entry time constants for free C subunit were similar (25 ± 11 min [n = 6] and 28 ± 6 min [n = 13] respectively). These results are also consistent with the finding that C subunits directly injected into the nucleus are seen to exit with a similar time course (Fantozzi *et al.*, unpublished data). Thus, free C subunit can freely exchange between the nucleus and cytoplasm in a manner independent of the cAMP concentration.

DISCUSSION

Our findings are consistent with C subunit entering the nucleus by diffusion. The degree of nuclear localization upon cytoplasmic microinjection of labeled C subunit is quantitatively similar to other macromolecules, 10-kDa dextran, and STI (Figure 1), which are accepted as entering the nucleus by diffusion (Peters, 1986; Breeuwer and Goldfarb, 1990). All three proteins are about twice as bright in the nucleus as in the cytosol, an effect that may be explainable by a higher quantum efficiency of the fluorescein tag in the nucleus. Alternatively, much of the apparent volume of the cytoplasm may actually not be accessible to solutes, perhaps because of organelles or cytoskeletal elements below light microscopic resolution (Horowitz and Paine, 1976). Increasing the size of the C subunit to 65 kDa by fusing C subunit to GST prevented translocation of C-GST (Figure 2) into the nucleus. This corresponds with the ≈40-kDa cutoff value for proteins that enter the nucleus by diffusion. Proteins much larger than GST, such as 240-kDa phycoerythrin, are dragged into the nucleus when coupled to a protein such as nucleoplasmin containing an effective NLS (Schultz and Peters, 1986). The inability of C subunit to drag GST into the nucleus is consistent with lack of a functional NLS. Rupture of the nuclear membrane, a procedure that should short-circuit active transport, did not markedly alter the nuclear to cytoplasmic distribution of free C subunit (Figure 3), providing additional evidence consistent with nuclear entry by diffusion. The inability to slow C subunit nuclear import by competition either with actively transported histone H1 or with excess C itself (Figure 4) is further evidence in favor of nuclear import by diffusion rather than a saturable transport mechanism. Depletion of energy or cooling cells blocked active nuclear import of ss-HSA but did not block nuclear entry of C subunit or STI (Figure 5). Finally, site-directed mutagenesis of single lysine residues (K189T and K192T), located at a possible nuclear localization signal sequence (189–192 KRVK) with homology to the large SV40 large T-antigen signal sequence, did not hinder nuclear localization of C subunit (Fantozzi *et al.*, 1992), a result in good agreement with the GST-C fusion protein remaining cytoplasmic.

Movement of C subunit into the nucleus by diffusion is also consistent with the anchorage-release model (reviewed by Nigg, 1990) for regulated compartmentation of signaling proteins such as NF-κB, the glucocorticoid receptor, and PKA. In this model inactive protein remains cytosolic whereas active protein continually



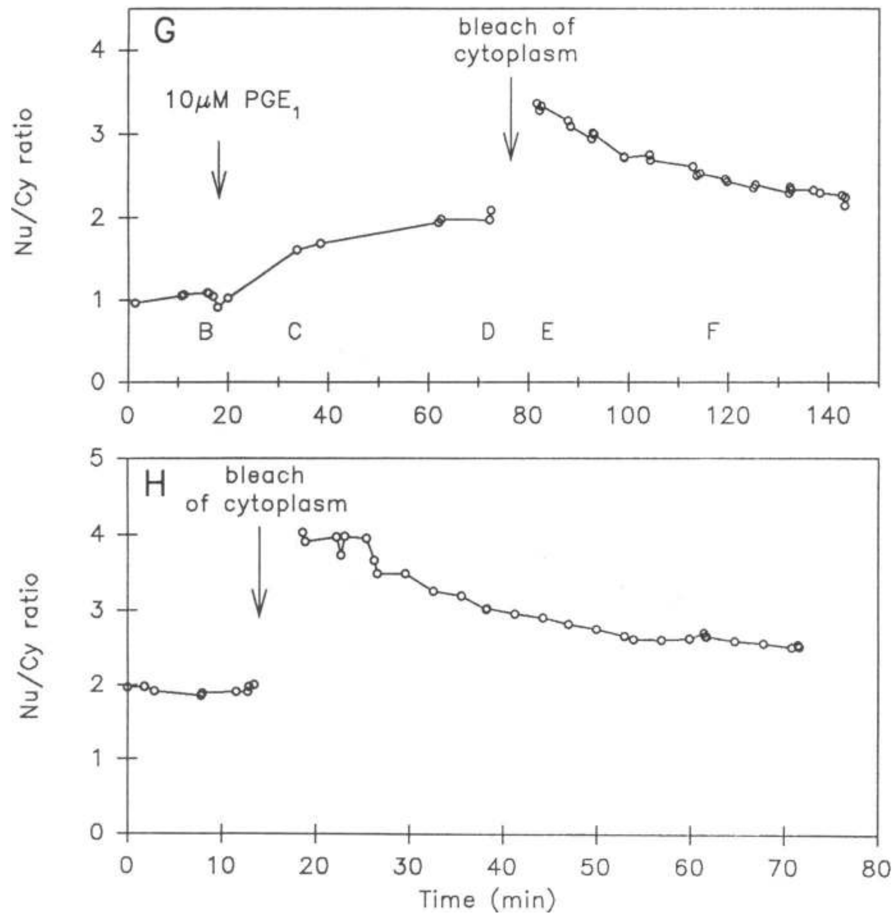


Figure 6. Exit of C subunit from the nucleus is revealed when cytoplasmic C subunit is photobleached. Fluorescence micrographs depicting fluorescence of FITC-labeled C subunit just after injection (A), 30 min after injection (B), after 17 min of treatment with 10 μM PGE_1 (C), after 56 min in PGE_1 (D), 5 min after photobleaching the cytoplasm (E), and 40 min after photobleaching the cytoplasm (F). The time course of changes in the nuclear to cytoplasmic brightness ratios after cytoplasmic photobleaching is shown for a cell initially injected with either FlCRhR (G) or free FITC-labeled C subunit (H). The cell shown in G is the same cell as shown in A–F; the time points corresponding to images B–F are indicated in G. The FlCRhR-injected cell was incubated with 10 μM PGE_1 to dissociate the PKA holoenzyme complex. In both experiments (A–G and H) C subunit had equilibrated between the nucleus and cytoplasm before cytoplasmic photobleaching.

shuttles between the cytoplasm and the nucleus. The cytoplasmic anchoring mechanism for PKA is based primarily upon the large size of PKA holoenzyme preventing passage through the nuclear pore, because the majority of microinjected PKA holoenzyme is diffusible (Harootunian, unpublished data). In addition to characterizing the mechanism by which C subunit enters the nucleus, we have directly shown that C subunit moves between the nucleus and cytoplasm in either direction at high effective [cAMP], a result also in agreement with the anchorage release model for regulated compartmentation of signaling proteins. Thus, the C subunit of PKA appears to be one member of a class of proteins that continually shuttle between the nucleus and cytoplasm such as HSP-70–related *Xenopus* heat shock proteins (Mandell and Feldherr, 1990), nucleolar proteins (Borer *et al.*, 1989; Meier and Blobel, 1990), and the progesterone receptor (Guiochon-Mantel *et al.*, 1991). Unlike C subunit, each of the above shuttling proteins is thought to enter the nucleus by active transport, because the proteins can carry other normally cytoplasmic proteins or microinjected antibodies into the nucleus. The mechanisms by which other shuttling proteins exit the nucleus are different; the progesterone receptor is thought to exit by passive diffusion (Guiochon-Mantel *et al.*, 1991), whereas the HSP-70–related heat shock proteins are thought to exit by active transport (Mandell and Feldherr, 1990). It is likely that the C subunit exits the nucleus by passive diffusion because the exit time is the same as the entry time (Figure 6).

In retrospect, the fact that the C subunit of PKA can exchange between the cytoplasm and the nucleus makes biological sense because PKA activity is essential in *both* the cytoplasm and the nucleus, whereas transcription factors such as NF-κB and progesterone receptors, which enter the nucleus by regulated active transport, have no obvious cytoplasmic functions. Cytoplasmic functions regulated by cAMP include glycogen breakdown in adipocytes, phosphorylation of ion channels in excitable cells, cytoskeletal motility (Lamb *et al.*, 1988, 1989; Sammak *et al.*, 1992), and secretion in a wide variety of cell types. The primary nuclear function of C subunit is regulation of genes via phosphorylation of the nuclear (Waeber and Habener, 1991) transcription factors of the CREB/ATF family. Microinjection of purified C subunit is sufficient to stimulate phosphorylation of CREB (Meinkoth, unpublished data) and induce CRE-regulated gene expression (Riabowol, 1992). Diffusion of the C subunit in and out of the nucleus links the cytoplasmic and nuclear functions of cAMP via a simple and reliable, though noninstantaneous (Bacskai *et al.*, 1993; Hagiwara *et al.*, 1993), mechanism, which must be understood before considering possible modulation of such signal transmission.

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