cAMP-dependent protein kinase mediates a variety of cellular responses in most eukaryotic cells. Many of these responses are cytoplasmic, whereas others appear to require nuclear localization of the catalytic subunit. In order to understand further the molecular basis for subcellular localization of the catalytic subunit, the effect of the heat stable protein kinase inhibitor (PKI) was investigated. The subcellular localization of the catalytic (C) subunit was determined both in the presence and absence of PKI, by microinjecting fluorescently labeled C subunit into single living cells. When injected alone, a significant fraction of the dissociated C subunit localized to the nucleus. When co-injected with an excess of PKI, little of the C subunit localized to the nucleus, suggesting that accumulation of catalytic subunit in the nucleus requires either enzymatic activity or a nuclear localization signal. Inactivation of the catalytic subunit in vitro by treatment with N-ethylmaleimide did not prevent localization in the nucleus, indicating that enzymatic activity was not a prerequisite for nuclear localization. In an effort to search for a specific signal that might mediate nuclear localization, a complex of the catalytic subunit with a 20-residue inhibitory peptide derived from PKI (PKI(5–24)) was microinjected. In contrast to intact PKI, the peptide was not sufficient to block nuclear accumulation. In the presence of PKI(5–24), the C subunit localized to the nucleus in a fashion analogous to that of dissociated, active C subunit despite evidence of no catalytic activity in situ. Thus, nuclear localization of the C subunit appears to be independent of enzymatic activity but most likely dependent upon a signal. The signal is apparently masked by both the regulatory subunit and PKI but not by the inhibitory peptide.

A fundamental cellular response to environmental stimuli

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The abbreviations used are: R, regulatory subunit of cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; CRE, cAMP response element; CREB/ATF, cAMP response element binding protein; PKI, thermostable inhibitor of cAMP-dependent protein kinase; PKI(5–24), 20-residue peptide comprising residues 5–24 of PKI; FITC, fluorescein 5-isothiocyanate; NEM, N-ethylmaleimide.

Effect of the Thermostable Protein Kinase Inhibitor on Intracellular Localization of the Catalytic Subunit of cAMP-dependent Protein Kinase*

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PKI Impairs Nuclear Localization of Catalytic Subunit

EXPERIMENTAL PROCEDURES

Materials—Fluorescein 5-isothiocyanate (isomer “F”; FITC) was obtained from Molecular Probes (Eugene, OR). Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was synthesized by the Peptide and Oligonucleotide Facility, University of California, San Diego. A 20-residue inhibitory peptide comprising residues 5-24 of PKI (PKI 5-24) was synthesized by Coast Scientific (La Jolla, CA) and subsequently purified by high performance liquid chromatography. All other chemicals were of reagent grade or the highest quality available commercially. Centricon microconcentrators were obtained from Amicon.

Preparation of Proteins and Peptides for Microinjection—Recombinant mouse catalytic subunit was purified from Escherichia coli as described (25), followed by fast protein liquid chromatography. The protein was labeled with FITC, as described previously, under conditions that led to minimal loss of activity (20). Samples (1 ml) were chromatographed at 4 °C through Sephadex G-25 (5-ml column) previously equilibrated with 5 mM sodium phosphate (pH 7.4), 100 mM KCl and then dialyzed against 4 liters of the same buffer at 4 °C for at least 12 h. Enzyme solutions were sterilized by filtration through 0.22-μm filters and concentrated by centrifugation using microconcentrators. The final concentration of catalytic subunit ranged from 8 to 10 mg/ml. Activity was measured with a synthetic peptide, Kemptide, using a coupled spectrophotometric method (26). Protein concentration was determined by measuring absorbance at 280 nm following correction for the contribution of FITC. When analyzed by Coomassie Blue staining following polyacrylamide gel electrophoresis carried out in the presence of sodium dodecyl sulfate, the protein appeared as a single band. Coincident fluorescence was detected by irradiating the gel at 300 nm. Recombinant PKI was purified as described (27) and quantitated by titration against the C subunit. PKI(5-24) was lyophilized five times from distilled H2O, titrated to pH 7.4, and quantitated by amino acid analysis and titration against the C subunit.

Inactivation of FITC-labeled Catalytic Subunit with N-Ethylmaleimide (NEM)—Labeled protein (1 mg/ml) was exchanged into 10 mM phosphate buffer (pH 7.0) by chromatography through Sephadex G-25 at 4 °C. Sulphydryl groups were modified using 0.5 mM NEM (28). Samples were incubated for 35 min, and inactivation was 97% or greater. The specific activity of control C subunit did not change during this time. Proteins were processed for microinjection as described above.

Cell Culture and Microinjection—Rat-2 CRE fibroblasts were plated on coverslips and grown to 50% confluence. Logarithmically growing cells were injected under serum-starved conditions. To correlate nuclear localization with catalytic activity in situ, aliquots of the two preparations described above were also injected into serum-starved REF-52 fibroblasts. Under these conditions, active FITC-C subunit induced morphological changes (detected as cell rounding) within 30 min (Fig. 3, a and b). In contrast, all cells injected with FITC-S subunit and excess PKI remained flat, consistent with kinase activity being undetectable in the injection mixture (Fig. 3, e and f).

FIG. 1. PKI prevents the nuclear localization of the catalytic subunit. Rat-2 CRE fibroblasts were plated on coverslips and grown to 50% confluence. Logarithmically growing cells were injected with fluorescently labeled C subunit (60 μM) in the absence (a) or presence (b, 140 μM) of PKI and fixed after 60 min. The specific activity of the C subunit was 7.5 μmol·min⁻¹·mg⁻¹. Catalytic activity was not detected in sample (b).
These data suggest that localization of the C subunit within the nucleus requires either catalytic activity or an exposed signal that mediates nuclear accumulation.

Effect of Catalytic Activity on Nuclear Localization of the C Subunit—To evaluate whether nuclear localization requires catalytic activity, the FTTC C subunit was inactivated in vitro. The enzyme was treated with N-ethylmaleimide (CNEM) under conditions that led to loss of activity. Although the NEM-treated FTTC C subunit retained less than 3% of its original activity, it localized rapidly to the nucleus (Fig. 2b) in a manner that was indistinguishable from the untreated C subunit (CAct; Fig. 2a). To assess independently any residual enzymatic activity of CNEM, both CAct and CNEM were injected into serum-starved REF-52 cells. Under these conditions injection of CAct caused the cells to round within 30–60 min, indicative of catalytic activity in situ. In contrast, all cells injected with CNEM remained flat (data not shown). These results suggest that any individual C subunit molecule does not need to be active in order to localize in the nucleus.

PKI Masks a Nuclear Localization Signal—In order to define more precisely whether PKI masks a region of the C subunit that is required for nuclear accumulation and to better localize this site, analogous experiments were performed with PKI(5–24). This peptide contains nearly all of the inhibitory potency of intact PKI (19). The interactions of this peptide with the C subunit are defined very precisely by the crystal structure of a C.PKI(5–24) binary complex (31, 32). Due to the smaller size of PKI(5–24) relative to PKI, the C.PKI(5–24) complex provided an enzymatically inactive complex in which, potentially, more regions on the surface of the C subunit are exposed than in the binary complex with intact PKI.

Mixtures containing C subunit and a 60-fold molar excess of PKI(5–24) were prepared in which no phosphotransferase activity could be detected in vitro. REF-52 cells were synchronized by serum deprivation and injected with FTTC C in the absence or presence of PKI(5–24) (Fig. 3) so that both nuclear localization and the presence of residual catalytic activity could be evaluated. In the absence of PKI(5–24), the catalytic subunit induced morphological changes (cell rounding) in a large proportion (>85%) of the injected cells (Fig. 3, a and b). In contrast, >90% of the cells injected with the PKI(5–24):FTTC C mixture remained flat (Fig. 3c) and exhibited strong nuclear fluorescence (Fig. 3d). In contrast, cells injected with PKI:FTTC C (molar ratio of 2.3:1.0) remained flat and exhibited predominantly cytoplasmic fluorescence (Fig. 3, e and f). Thus, despite inhibition of catalytic activity in situ, PKI(5–24) did not impede the nuclear localization of the C subunit. These results are consistent with the hypothesis that a signal is necessary and sufficient for nuclear localization of the C subunit and suggest that PKI, but not PKI(5–24), prevents significant nuclear localization by masking that signal.

DISCUSSION

Previous studies demonstrated that a significant fraction of the dissociated catalytic subunit of cAMP-dependent protein kinase localized to the nucleus (20, 21, 33). Thus one potential role for the regulatory subunit of cAMP-dependent protein kinase appears to be as a cytoplasmic anchor for the catalytic subunit. PKI is the other known physiological inhibitor of the C subunit; given its smaller size (75 amino acids versus 758 in the R dimer), we evaluated its capacity to regulate the intracellular distribution of the dissociated C subunit. Mixtures of fluorescently labeled C subunit and excess PKI were introduced into the cytoplasm of living cells by microinjection. In contrast to cells injected with free C subunit, cells injected with C:PKI mixtures did not exhibit significant nuclear fluorescence.

In an effort to distinguish whether the C.PKI complex was excluded from the nucleus because a localization signal was masked or because it lacked enzymatic activity, FTTC C subunit was inactivated by treatment with N-ethylmaleimide. Although CNEM retained only minimal phosphotransferase activity in vitro, it localized readily to the nucleus in a manner analogous to that of active C subunit following microinjection. This occurred despite a lack of morphological changes in the injected cells, indicating that there was insignificant kinase activity in situ. These results establish that intrinsic kinase activity is not required for localization to the nucleus. An individual molecule need not be active in order to localize. However, this does not eliminate the possibility that a trace amount of catalytic activity may be sufficient to open a nuclear pore.

The mechanism(s) by which the C subunit localizes to the nucleus is unknown. Its size (∼40 kDa) does not preclude it from simply diffusing freely into the nucleus (limit, ∼50 kDa) (34). On the basis of size, in the absence of an exposed nuclear translocation signal, the holoenzyme would be excluded from the nucleus. However, the C.PKI complex could not be excluded on the basis of size. Since intrinsic activity per se is not required for localization, PKI must be masking a signal that is necessary either for entry into or trapping within the nucleus.

In order to localize further such a signal, the effect of a smaller fragment of PKI was investigated. This peptide, PKI(5–24), was shown previously to contain most of the inhibitory potential of intact PKI. Furthermore, the recent solution of the crystal structure of the catalytic subunit complexed with PKI(5–24) (31, 32) provides a detailed profile of the regions on the surface that are masked by the binding of PKI.
PKI Impairs Nuclear Localization of Catalytic Subunit

Fig. 3. PKI(5–24) does not prevent the nuclear localization of the C subunit. Quiescent rat embryo fibroblasts were injected with fluorescently labeled C subunit (60 μM) either alone (a, b) or in the presence of 3.6 mM PKI(5–24) (c, d) or 140 μM PKI (e, f) and fixed after 60 min. The specific activity of the C subunit was 21 μmol.min⁻¹.mg⁻¹. No catalytic activity was detected in samples c and e. Phase contrast photomicrographs are shown on the left and fluorescence photomicrographs on the right.

the inhibitor peptide. The results obtained with the FTTC:C:PKI(5–24) mixture are similar to those seen for the C subunit inactivated with NEM. No morphological effects indicative of kinase activity were observed in the injected cells; however, nuclear localization was apparent.

The difference between PKI and PKI(5–24) in their capacity to block nuclear localization of the C subunit provides some indication of where a signal might be located. A recent mapping of some of the surface recognition sites for the R subunit is also informative. PKI, PKI(5–24), and the R subunit all share a common consensus sequence that binds to the active site of the C subunit. The high affinity binding of PKI and PKI(5–24) is due to the amphipathic helix that lies proximal to this consensus site. In contrast, most of the region important for the high affinity binding of R lays on the surface that is immediately distal to the consensus site based on a genetic scanning of the yeast AMP-dependent protein kinase (35). Any portion of PKI that might complement this distal site is missing in PKI(5–24). Hence, we conclude that if there is a signal for nuclear localization within the C subunit, it very likely resides on this surface that is masked by the R subunit.

Although there is no single, strict consensus sequence for nuclear import, the prototypical nuclear localization signal is short, relatively basic, not confined to any particular region of the protein, not removed during localization, and occasionally greater than 1 in number (36). Only one sequence within the C subunit conforms to the consensus sequence generated from homologs of the SV40 large T antigen: residues 189–192, Lys-Arg-Val-Lys, versus consensus of Lys-Arg/Lys-X-Arg/Lys (37). This sequence is found within one of the signals deduced for human c-Myc (38, 39). Consistent with our model, this sequence is exposed in the binary complex of the C subunit with PKI(5–24) (31, 32). However, site-directed mutagenesis of individual lysine residues (K189T and K192T) of the C subunit did not impair nuclear localization. These results suggest that this sequence may not be involved in nuclear localization or at least is not sufficient for nuclear localization; some sequences that are very similar to the SV40 large T sequence fail in nuclear targeting assays (40, 41). Alternatively, impairing translocation may require more drastic mutagenesis, since the nuclear localization signal may consist of interdependent basic domains (40). We are now probing the structure more extensively in attempts to identify a signal.

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PKI Impairs Nuclear Localization of Catalytic Subunit