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Identification of a Signal for Rapid Export of Proteins from the Nucleus

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Summary

Active nuclear import of protein is controlled by nuclear localization signals (NLSs), but nuclear export is not understood well. Nuclear trafficking of the catalytic (C) subunit of cAMP-dependent protein kinase (cAPK) is critical for regulation of gene expression. The heat-stable inhibitor (PKI) of cAPK contains a nuclear export signal (NES) that triggers rapid, active net extrusion of the C-PKI complex from the nucleus. This NES (residues 35–49), fused or conjugated to heterologous proteins, was sufficient for rapid nuclear export. Hydrophobic residues were critical. The NES is a slightly weaker signal than the SV40 NLS. A sequence containing only residues 37–46, LALKLAGLDI, is also sufficient for nuclear export. This is an example of a protein-based NES having no obvious association with RNA. A similar sequence, LQLPPLERLTL, from Rev, an RNA-binding protein of HIV-1, also is an NES.

Introduction

Selective targeting of proteins within cells requires the macromolecules to bear sorting determinants. Signal sequences for protein transport into endoplasmic reticulum (ER), mitochondria, lysosomes, and chloroplasts (for reviews, see Walter and Johnson, 1994; Schwarz and Neupert, 1994; Kornfeld and Mellman, 1989; Smeekens et al., 1990) are well documented. The exchange of macromolecules between cytoplasm and nucleus is different from transport into other organelles because of the presence of stable pores in the nuclear envelope. Nuclear pore complexes, consisting of perhaps 1000 proteins in 60–100 separate species, provide aqueous channels with a functional diameter of 9–10 nm. Passage of small proteins (<40–60 kDa) through these pores can occur by diffusion, but the import of larger proteins (>40 kDa), as well as RNAs and some small proteins, is an active process: it is saturable, both temperature and ATP dependent, and causes substrate accumulation well beyond passive equilibrium. Active import of proteins into the nucleus requires the presence of nuclear localization signals (NLSs) (for reviews, see Dingwall and Laskey, 1992; Forbes, 1992; Garcia-Bustos et al., 1991; Nigg et al., 1991; Silver, 1991, and references therein). NLSs do not fit a tight consensus but generally fall into two classes: short basic sequences of four to seven amino acids (Kalderon et al., 1984) and longer bipartite sequences consisting of two stretches of basic amino acid sequences separated by ten less-conserved spacer amino acids (Robbins et al., 1991). Possession of NLSs, while probably necessary, is not sufficient to ensure nuclear import. The NLSs may be masked by binding to other domains or proteins (for reviews, see Silver, 1991; Nigg, 1990) and may be modulated by phosphorylation (Rihs et al., 1991). Active import of protein into the nucleus is believed to occur in two steps: binding to the cytoplasmic surface of the nuclear pore complex independent of ATP or GTP, followed by the energy-dependent translocation through the nuclear pore complex (Newmeyer and Forbes, 1988; Richardson et al., 1988). A number of cytosolic components have been shown by various methods and systems to be involved (Adam and Gerace, 1991; Moore and Blobel, 1992; for reviews, see Powers and Forbes, 1994; Melchior and Gerace, 1995, and references therein).

The same nuclear pores are also employed in the export of macromolecules such as RNA and protein (Dworetzky and Feldherr, 1988; Featherstone et al., 1988), but export is much less well understood than import. RNA export is probably a signal-mediated active process, because it is saturable and sensitive to low temperature, ATP depletion, and wheat germ agglutinin inhibition. Export of RNAs from the nucleus also seems to be dependent on the binding of other proteins (Bataille et al., 1990; Dargemont and Kuhn, 1992; Zasloff, 1983; for reviews, see Elliott et al., 1994; Izaurralde and Mattaj, 1995, and references therein). Several RNA-binding proteins have been suggested to be involved in RNA transport: either the transcription factor TFIIIA or ribosomal protein L5 is required for the nuclear export of 5S RNA (Guddat et al., 1990); the influenza virus protein M1 is necessary for nuclear export of viral RNA–protein complexes (Martin and Helenius, 1991); the human immunodeficiency virus type I Rev protein directly promotes the nuclear export of unspliced RNA (Fischer et al., 1994; Meyer and Malim, 1994); and Nup145p is required for nuclear export of messenger RNA (mRNA) (Fabre et al., 1994). It remains unclear whether the export signal for RNA export resides solely on RNA, or on protein, or both. The signal required for export of RNAs on RNA that has already been identified is the 5′ monomethylated cap structure for the export of mRNA and small nuclear RNA (Dargemont and Kuhn, 1992; Hamm and Mattaj, 1990).

Information on export of proteins from the nucleus is even more limited. A number of proteins are known to shuttle between nucleus and cytoplasm, some constitutively and others in response to regulatory signals. These include various nucleolar proteins, heat shock protein (hsp70), steroid hormone receptors, cAMP-dependent protein kinase (cAPK), components of ribonucleoprotein (RNP), and NLS-binding protein Nopp140 (reviewed by

Goldfarb, 1991; Laskey and Dingwall, 1993, and references therein). They might contribute to coordinating nuclear and cytoplasmic activities in the cell. An important question has been whether exit of shuttling proteins from the nucleus represents passive diffusion or an active process requiring an export signal. One study on proteins shuttling out of Xenopus oocyte nuclei suggested that specific domains are required only for nuclear retention and that export is a default process not requiring a signal (Schmidt-Zachmann et al., 1993; reviewed by Laskey and Dingwall, 1993). However, this result could not exclude the possibility that some proteins exit from the nucleus by a facilitated, energy-dependent process, as implicated by the temperature-dependent exit of pre-mRNA-binding protein (heterogeneous nuclear RNP, or hnRNP) from the nucleus (Piñol-Roma and Dreyfuss, 1992) and the possible involvement of NLS in mediating the nuclear export of some shuttling proteins (Guiochon et al., 1994). The observation that the catalytic subunit (C) of cAPK is actively exported from the nucleus by its physiological inhibitor, heat-stable protein kinase inhibitor (PKI), first clearly demonstrated the presence of active nuclear export of proteins that exhibit no obvious RNA binding (Fantozzi et al., 1994; Wen et al., 1994).

cAPK exists predominantly as an inactive cytoplasmic holoenzyme consisting of two regulatory (R) and two catalytic (C) subunits. Following an increase in intracellular cAMP, the R subunits bind cAMP, resulting in holoenzyme dissociation and release of free active C subunit, which can then enter the nucleus by passive diffusion (Adams et al., 1991; Harootunian et al., 1993; Meinkoth et al., 1990). PKI alone can enter the nucleus and causes C subunit to leave the nucleus (Adams et al., 1991; Fantozzi et al., 1994). The C–PKI complex exits the nucleus at a rate much faster than the free C subunit equilibrates by passive diffusion, and the final distribution of C–PKI between cytoplasm and nucleus is far from passive equilibrium. PKI does not act by altering the binding of C subunit to nuclear or cytoplasmic constituents, because photobleaching measurements show that the lateral mobility of C subunit is the same in the nucleus and in the cytoplasm regardless of the presence or absence of PKI. Also, simple puncture of the nuclear envelope immediately allows the complex back into the nucleus (Fantozzi et al., 1994). The export of C–PKI is temperature and ATP dependent but tolerates a large increase in the size of either the C subunit or PKI. C–PKI complexes in which C is fused to glutathione S-transferase (GST), 140 kDa total, or in which PKI is fused to maltose-binding protein (MBP) or GST, 90 kDa and 140 kDa total, respectively, greatly exceed the size limit for diffusion through nuclear pores, yet are rapidly exported. These results suggested that the C–PKI complex exposes a nuclear export signal (NES) triggering active net extrusion. Further studies showed that the fusion of PKI with other proteins, such as GST, resulted in its rapid exit from the nucleus even without C, suggesting that the NES resides solely on PKI (Wen et al., 1994). But the nature and composition of this signal were not defined. By constructing chimeric fusion proteins, we have now located the region of PKI that encodes the NES. Site-directed mutagenesis subsequently revealed which amino acids are most crucial for this signal.

Results

PKIα(35–49) Is Sufficient to Direct GST from Nucleus to Cytoplasm

In previous studies, we showed that PKI carries an NES and that this signal is likely to reside on the conserved residues between PKIα and PKIβ1, since both forms of PKI can export C subunit from the nucleus (Wen et al., 1994). PKIα and PKIβ1 share 41% identity (Van Patten et al., 1991). Amino acid sequence alignment (Figure 1) reveals that three regions are well conserved. Both PKIα and PKIβ1 contain a pseudosubstrate sequence, RRNAI, that mimics the substrate sequence and interacts with C subunit with high affinity. However, the PKI inhibitor peptide (5–24) (reviewed by Walsh et al., 1990), including the pseudosubstrate site, is unable to exclude C subunit from the nucleus, even though it binds C subunit tightly (Fantozzi et al., 1994). This suggested that the other two parts of PKI might be involved in nuclear export. One region at the N-terminus of PKI, from Thr-1 to Glu-4, may function as an N-capping motif of an α helix as demonstrated by nuclear magnetic resonance (NMR) studies (A. Padilla et al., personal communication). The other is a hydrophobic residue–rich region of unknown function in the middle of PKI, from Leu-37 to Thr-49. To locate the NES, PKIα was systematically truncated from both the N- and the C-terminus. All deletion mutants were fused with GST (54 kDa as dimer) as chimeric proteins. All the fusion proteins were expressed in Escherichia coli and purified by use of glutathione–agarose columns. The purified proteins were then labeled with fluorescein isothiocyanate (FITC) and microinjected into either the cytoplasm or the nucleus of REF52 fibroblasts. Guinea pig immunoglobulin G (IgG), which cannot traverse the nuclear envelope, was coinjected to identify the injection sites. As shown previously, GST alone spreads throughout the cell irrespective of the initial injection site (Wen et al., 1994). However, when it is fused to either the N-terminus (GST–PKI) (Wen et al., 1994) or the C-terminus (PKI–GST) (Figure 2A) of PKIα, the

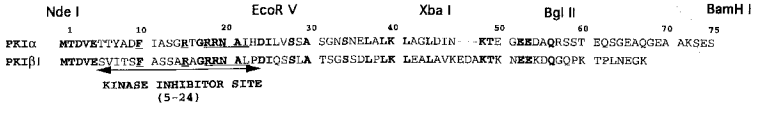

Figure 1. Sequence Comparison between PKIα and PKIβ1

Identical residues between PKIα and PKIβ1 are in bold. Residues important for high affinity binding to C subunit are underlined. The partial restriction enzyme map of *PKI* cDNA (Thomas et al., 1991) is shown above the amino acid sequence.

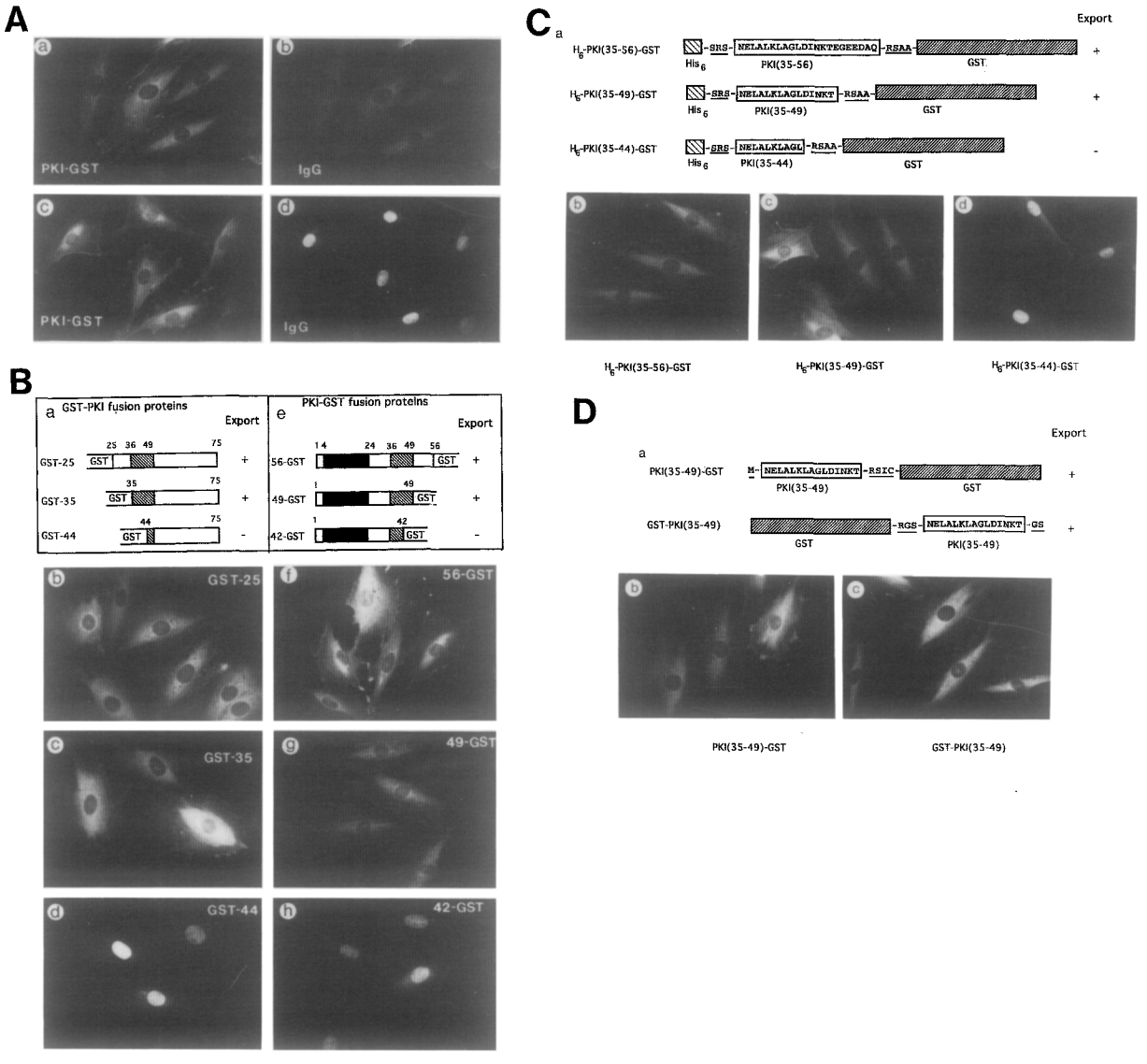

Figure 2. PKIα(35–49) Is Sufficient to Direct GST from Nucleus to Cytoplasm

(A) PKI–GST fusion protein is excluded from the nucleus. The C-terminus of full-length PKI was fused to the N-terminus instead of the conventional C-terminus of GST. The fusion protein was expressed in E. coli, purified on a glutathione–agarose column, and labeled with FITC. The FITC-labeled protein was then coinjected with IgG either into the cytoplasm (a) or the nucleus (c) of REF52 cells. Cells were fixed 30 min after injection and stained for the coinjected IgG with an aminomethylcoumarin acetate–conjugated anti-guinea pig antibody (b and d).

(B) Nuclear export activity is localized to the region of PKIα from residue 35 to residue 49. (a) and (e) show schematic representations of the GST–PKI ([a], GST-25, GST-35, and GST-44) and PKI–GST ([e], 56-GST, 49-GST, and 42-GST) constructs. The N-terminal conserved region is labeled 1–4, the closed regions represent the PKI inhibitor peptide (5–24), and the hatched regions represent the hydrophobic residue–rich region (37–49). The subcellular localizations of fusion proteins were examined 45 min after injection, as described in (A). The nuclear export capacity of these proteins is summarized in (a) and (e), where plus indicates fusion proteins that are extruded from the nucleus following nuclear injection and minus indicates fusion proteins that remain in the nucleus following nuclear injection. Fluorescence images showing the fate of fusion proteins after nuclear injection are shown in (b) (GST-25), (c) (GST-35), (d) (GST-44), (f) (56-GST), (g) (49-GST), and (h) (42-GST).

(C) PKIα(35–49) is sufficient for nuclear export. (a), schematic representation of constructs. The oligonucleotides corresponding to sequence (35–56, 35–49, or 35–44) were introduced into the site between polyhistidine and GST. Amino acids derived from PKIα(35–49) are boxed; flanking sequence from cloning sites are underlined. The subcellular localization of these fusion proteins was determined and summarized as described in Figure 2B. Fluorescence emanating from the fusion proteins is shown in (b) (H_6–PKIα(35–56)–GST), (c) (H_6–PKIα(35–49)–GST), and (d) (H_6–PKIα(35–44)–GST).

(D) The position of the PKIα(35–49) sequence in the fusion proteins has little effect on nuclear export. The chimeras were formed by fusing PKIα(35–49) either at the beginning (PKIα(35–49)–GST), or at the end (GST–PKIα(35–49)) of GST (a). The subcellular localization of these two fusion proteins is shown in (b) (PKIα(35–49)–GST) and (c) (GST–PKIα(35–49)).

chimeric proteins are excluded from the nucleus following nuclear injection and remain in the cytoplasm for at least 45 min following cytoplasmic injection. However, the fates of the fusion proteins containing truncations in PKIα varied after nuclear injection (Figure 2B). Both PKIα(25–75) (Figure 2Bb) and PKIα(35–75) (Figure 2Bc), with conserved residues 37–49 but not 1–4, were sufficient to target the GST from the nucleus to the cytoplasm, whereas PKIα(44–75) (d), with partial hydrophobic residue conserved regions, was unable to export GST. Both PKIα(1–56) (Figure 2Bf) and PKIα(1–49) (Figure 2Bg), but not PKIα(1–42) (Figure 2Bh), were sufficient to export fusion proteins from the nucleus following nuclear injection. Taken together, these results suggested that the NES is likely to locate on the region of PKIα between 35–49.

To confirm whether PKIα(35–49) is sufficient to export protein from the nucleus, sequences corresponding to PKIα residues 35–56, 35–49, or 35–44 were inserted between the N-terminal polyhistidine tag (about 23 amino acids) and the GST protein. The first two fusions but not the third left the nucleus following nuclear injection (Figure 2C), while all remained in the cytoplasm following cytoplasmic injection (data not shown). Thus, PKIα residues 35–49 are likely to contain the NES. This sequence was also effective when fused to the N- or C-terminus of GST (Figure 2D). Since the different flanking regions of the GST fusion proteins did not affect the cellular distribution following nuclear injection, the NES must come solely from PKIα(35–49). Therefore, like an NLS, a single short peptide is also able to direct protein export from the nucleus.

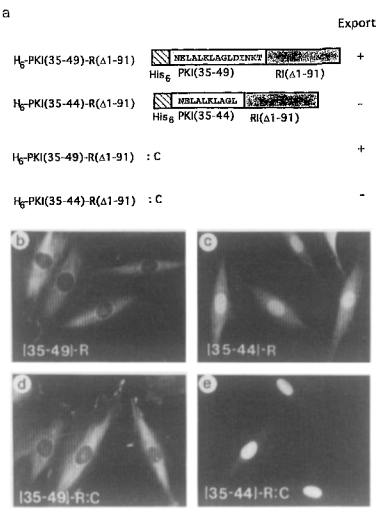

Figure 3. PKIα(35–49) Is Also Sufficient to Export Monomer R^I Mutant and Its Complex with C Subunit from the Nucleus

All the constructs are depicted schematically in (a). Oligos of PKIα(35–49) or PKIα(35–44) were fused with deletion R^I subunit mutant (R(Δ1–90)) where the dimerization domain from 1–91 was deleted. These fusion proteins were expressed in E. coli and purified by using a Ni^{2+} chelate column. The purified proteins were either labeled with FITC or complexed with FITC-labeled C subunit and then injected into the nucleus. Cells were fixed and stained for coinjected IgG 45 min after injection. (b) and (d) show the distributions of the fusion protein H_6–PKIα(35–49)–R^I(Δ1–91) alone and its complex with the C subunit, respectively. (c) and (e) show the distributions of the fusion proteins (H_6–PKIα(35–44)–R^I(Δ1–91) alone and complexed with C subunit, respectively.

PKIα(35–49) Is Also Able to Export Deletion R Subunit and Its Complex with C Subunit from the Nucleus

We next assessed whether residues 35–49 of PKIα are sufficient to export proteins other than GST from the nucleus. The R^I subunit of cAPK lacking the dimerization domain from 1 to 91 was chosen because of its relevance to cAMP signaling and potential usefulness. Its subcellular distribution is dependent on complexation: alone, it is small enough to cross the nuclear envelope, but it is too large once it binds the C subunit (Herberg et al., 1994). As shown in Figure 3, insertion of PKIα(35–49) between the N-terminal polyhistidine tag and R^I(Δ1–91) was sufficient to cause nuclear export (Figure 3b), whereas the fusion protein stayed in the cytoplasm following cytoplasmic injection (data not shown). When this fusion protein was bound to FITC-labeled C subunit, the complex was still exported and excluded from the nucleus (Figure 3d). In contrast, the analogous fusion H_6–PKIα(35–44)–R^I(Δ1–91) by itself equilibrated between the cytoplasm and the nucleus regardless of injection site (Figure 3c), and its complex with C subunit was unable to leave the nucleus following nuclear injection (Figure 3e). Therefore, PKIα(35–49), unlike PKIα(35–44), is capable of excluding a medium-sized protein, R^I(Δ1–91) (41 kDa), from the nucleus and directing a larger protein complex (80 kDa with C subunit) from the nucleus to the cytoplasm. These results confirmed that this region encodes an NES.

Hydrophobic Residues Are Necessary for Nuclear Export

Once the sequence of an NES had been approximately defined, the contributions of individual amino acids were then characterized. All the conserved residues were replaced with alanines either individually or together in full-length PKIαs. These mutant PKIαs were purified, complexed with FITC-labeled C subunit, further purified on a gel filtration column, and microinjected into either the cytoplasm or the nucleus of REF52 cells. IgG was coinjected to document the sites of injection and absence of leakage. Mutation of conserved hydrophilic residues of PKIα such as Glu-36, Lys-40, Gln-47, Lys-48, and Thr-49 to alanine had little impact on the export of the complex of FITC-labeled C subunit with PKIα. However, when hydrophobic residues such as the leucines at positions 37, 39, 41, and 44 or the isoleucine at 46 were replaced with alanines, significant effects were observed (Figure 4A). When Leu-37, Leu-39, and Leu-41 were simultaneously changed to alanines, the mutant was no longer able to export C subunit from the nucleus. But when nearby pairs of leucines (either 37, 39 or 39, 41) were mutated to alanines, export persisted. Interestingly, when the leucines at 37 and 41 were changed to alanines, export was dramatically impaired. This is consistent with the secondary structure of PKIα(35–44) determined in solution by NMR, which

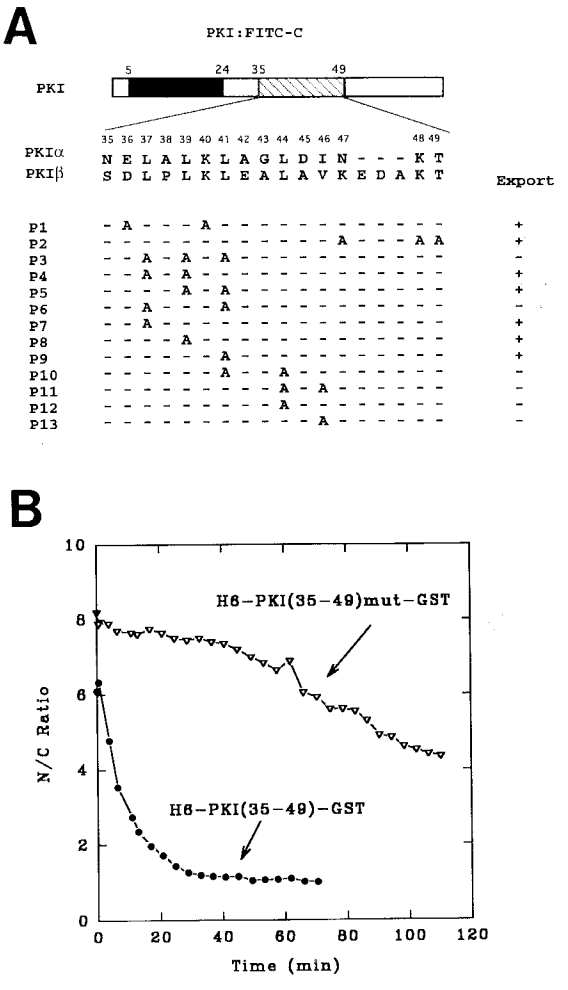

Figure 4. Hydrophobic Residues Are Critical for Nuclear Export

(A) Mutant PKIαs where the hydrophobic residues in the region of PKIα(35–49) were replaced with alanines are not able to export FITC-labeled C subunit from the nucleus. Mutations were introduced into full-length PKIα as indicated in the schematic representation. The mutants are named on the left. All mutant PKI proteins were purified and complexed with FITC-labeled C subunit. The complex was then coinjected with IgG into the nucleus. The cells were then fixed 30 min after injection. The export ability of these proteins is summarized.

(B) Mutant forms of PKIα(35–49) where the hydrophobic residues were replaced with alanines are defective in exporting GST from the nucleus. The mutant form of PKIα(35–49), where Leu-41 and Leu-44 were replaced with alanines, was introduced in the middle (H_6–PKIα(35–49)$_{mut}$–GST) of the fusion protein. The fusion proteins containing wild-type PKIα(35–49) and mutant PKIα(35–49) were injected into the nucleus. The distribution of both proteins were assessed in living cells as a function of time by quantitative fluorescence imaging.

indicates that residues 35–44 form an α helix with residues 37 and 41 on the same side, forming a hydrophobic surface (A. Padilla et al., personal communication). When Leu-37, Leu-39, or Leu-41 was individually changed to alanine, no significant effects on the export of C subunit from the nucleus were observed. However, replacement of Leu-44 and Ile-46 with alanine, either individually or together, significantly impaired the ability to exclude the C subunit from the nucleus. Mutation of Ile-46 seemed to give the more dramatic effect. As might be expected, replacement of both Leu-41 and Leu-44 by alanines also blocked export (Figure 4A). Therefore, the residues most important for the NES are Leu-37, Leu-41, Leu-44, and Ile-46, with Leu-39 possibly playing a minor role.

To confirm the importance of these hydrophobic residues, similar mutations were introduced into fusions of GST with shorter pieces of PKIα. Single mutations L44A or I46A or double mutation L41A, L44A prevented nuclear export of GST fused to PKIα(25–75) (data not shown). Likewise, double mutation L41A, L44A significantly impaired nuclear export when applied to a PKIα fragment (35–49) placed either at the N-terminus of the fusion protein or between a polyhistidine tag and GST (data not shown). Quantitative fluorescence imaging of the FITC-labeled proteins as a function of time after nuclear injection showed that the protein with a mutant NES was exported much less quickly and completely than protein with wild-type NES (Figure 4B). This further documented the specificity of the identified NES encoded by PKI residues 35–49.

Chemical Cross-Linking to Synthetic PKIα(35-49) Causes Extrusion of Bovine Serum Albumin and IgG from the Nucleus

Nuclear localization signals are powerful and autonomous enough to trigger nuclear import even when they are chemically conjugated rather than genetically fused to the transport substrate. To see whether the NES is equally autonomous, the peptide P(35–49), corresponding to PKIα(35–49), and the mutant P(35–49)$_{mut}$, with Leu-41 and Leu-44 replaced by alanines, were synthesized and purified by high pressure liquid chromotography (HPLC). Both peptides were designed with a C-terminal cysteinamide so that they could be coupled to carrier proteins by using the heterobifunctional cross-linking reagent succinimidyl *trans*-4-(N-maleimidylmethyl)cyclohexane-1-carboxylate (SMCC). This reagent linked the C-terminal thiol on the peptide with lysine side chains on the marker proteins. Bovine serum albumin (BSA) and rabbit IgG were chosen as markers, because they are well known to be too large to pass through nuclear pores unless actively transported (Borer et al., 1989; Goldfarb et al., 1986; Guiochon et al., 1991; Lanford et al., 1986). Peptide-decorated proteins P(35–49)–BSA, P(35–49)$_{mut}$–BSA, and P(35–49)–IgG contained 5–10 peptides per molecule of BSA and 1–5 peptides per molecule of IgG as estimated by the molecular weight changes compared with those of uncoupled carrier proteins. P(35–49)–BSA and P(35–49)$_{mut}$–BSA were labeled with FITC either before or after the coupling. Rabbit IgG was detected with FITC-conjugated antibody against rabbit IgG. After nuclear injection, proteins decorated with wild-type peptide were found in the cytoplasm after 45 min (Figures 5a and 5c). When injected into the cytoplasm, they remained in the cytoplasm (data not shown). In contrast, uncoupled BSA or IgG or BSA conjugated to the mutant peptide was unable to cross the nuclear envelope in either direction, and each remained in its initial injection site (Figures 5b and 5d). These results show that PKIα(35–

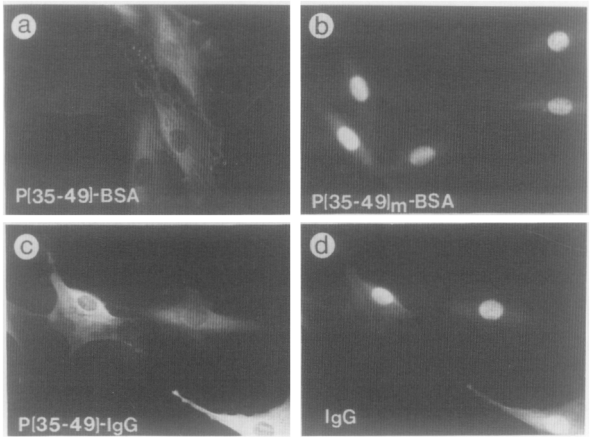

Figure 5. A Synthetic Peptide Is Able to Direct BSA and IgG from the Nucleus to the Cytoplasm

A peptide corresponding to the sequence of PKIα(35–49) was synthesized and conjugated to BSA (a) and IgG (c) with the bifunctional cross-linking reagent SMCC. The distribution of BSA conjugated with mutant peptide, where the Leu-41 and Leu-44 were replaced with alanines, is shown in (b). The distribution of coinjected uncoupled IgG with peptide-conjugated IgG is shown in (d).

49) is an effective NES even when attached by nonphysiological cross-links to marker proteins far too large to diffuse through the nuclear pore.

NES Is a Slightly Weaker Signal Than NLS

The identification of the NES raises an interesting question: what will be the cellular fate of a protein containing both an NES and an NLS? Fusion proteins were constructed as shown in Figure 6A, with an NES of PKI and an NLS of SV40 large T antigen fused respectively to the N- and C-terminus of GST (Figure 6Aa). As usual, the distribution of these proteins was determined after microinjection of the FITC-labeled proteins into the cytoplasm (Figure 6Ab) and nucleus (Figure 6Ac). The fusion protein bearing both wild-type NES and NLS was found both in the nucleus and cytoplasm (Figures 6Ab and 6Ac), but the nuclear fluorescence was brighter than cytoplasmic fluorescence (Figure 6A). The final distribution was qualitatively similar, regardless of the initial injection site. Quantitative imaging showed a final nuclear/cytoplasmic intensity ratio of 3.6 ± 1.2, only slightly higher than the ratios (1.5–2.5) for macromolecules such as 10 kDa dextran or free C subunit known to equilibrate passively across the nuclear envelope (Harotounian et al., 1993) (Figure 6B). Because the same final nuclear/cytoplasmic ratio is quickly attained whether the NES–GST–NLS fusion protein is injected into the nucleus (initial nuclear/cytoplasmic ratio, >10) or into the cytoplasm (initial ratio, <1), the protein can clearly cross the nuclear envelope in either direction and is probably still shuttling rapidly in a futile cycle at steady state. As expected, when a wild-type NLS was pitted against a mutant NES in which alanines replaced the leucines corresponding to PKIα positions 41 and 44 (Figure 6Ad), the fusion localized essentially exclusively into the nucleus following either nuclear (Figure 6Af) or cytoplasmic (Figure 6Ae)

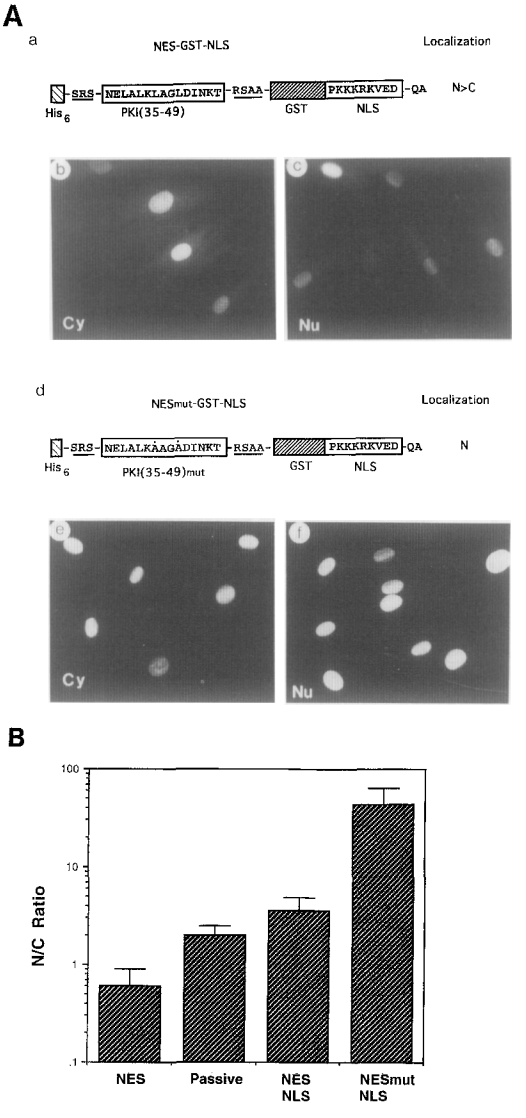

Figure 6. Fusion Protein Containing both NES and NLS Is Localized in Both Nucleus and Cytoplasm with Slightly Stronger Nuclear Fluorescence

(A) (a) and (d) are schematic representations of the fusion proteins. The oligonucleotides that specify the SV40 large T antigen NLS were introduced into the C-termini of wild-type NES–GST (H_6–PKIα(35–49)–GST) (a, b, and c) and mutant NES–GST (H_6–PKIα$(35–49)_{mut}$–GST) (d, e, and f). The fusion proteins were injected either in the cytoplasm (b, e) or in the nucleus (c, f). The injected cells were fixed 45 min after injection. The results are summarized. N, nuclear; C, cytoplasmic.

(B) Nuclear/cytoplasmic fluorescence intensity ratios are plotted for NES-containing proteins (e.g., the complex of FITC-labeled C subunit with PKI), proteins passively equilibrated between nucleus and cytoplasm (10 kDa dextran, C subunit of cAPK), protein containing both NES and NLS (NES–GST–NLS), and protein containing defective NES and functional NLS (NES_{mut}–GST–NLS). All the ratios were measured by fluorescence imaging 45 min after injection.

injection. The quantitative nuclear/cytoplasmic ratio was 44 ± 20 (Figure 6B). These results suggest that an intact NES is almost but not quite as strong as an NLS and confirm the importance of Leu-41 and Leu-44 in the NES.

Sequence Containing Only the Ten Residues 37-46 Is Sufficient for Nuclear Export

Knowing that hydrophobic but not hydrophilic residues are crucial, we then investigated whether the sequence 37–46 of PKIα, which contains all the critical hydrophobic residues but not the flanking hydrophilic residues, is sufficient for nuclear export. The sequence corresponding to the region 37–46 of PKIα was fused to the C-terminus of GST, and the chimeric protein was then microinjected into either the nucleus or the cytoplasm. The fusion protein was excluded from the nucleus 30 min after injection (Figure 7A). Thus, a sequence as short as 10 residues encodes an NES, which is separated from the PKI inhibitory region (5–24). Therefore, PKI utilizes two distinct domains to achieve different biological functions, kinase inhibition and nuclear export (Figure 8).

The Effector Domain of HIV-1 Rev Protein Is Also Sufficient to Export GST Fusion Protein from the Nucleus

Human immunodeficiency virus type 1 (HIV-1) Rev protein is an RNA-binding protein containing two essential functional domains. The N-terminal domain is necessary and sufficient for RNA binding, multimerization of protein, and nuclear localization (Bohnlein et al., 1991; Hammerschmid et al., 1994; Malim et al., 1989). The C-terminal domain is the effector domain, which is rich in leucine residues (Malim et al., 1991; Venkatesh and Chinnadurai, 1990) (Figure 8). The effector domain has been shown to be

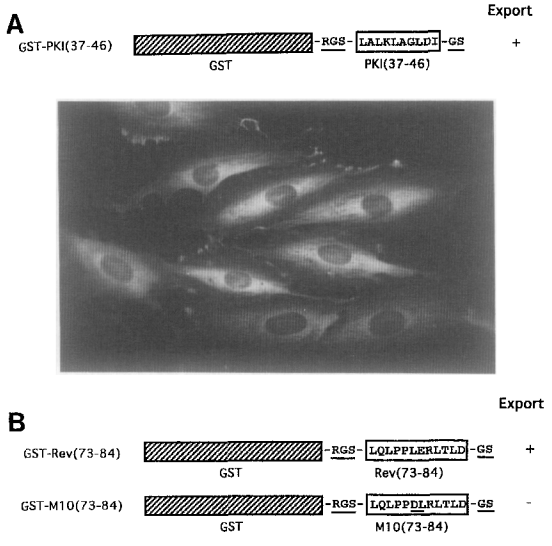

Figure 7. The Effector Domain of HIV-1 Rev Protein Shares Some Similarity to NES of PKI

(A) Ten residues of PKIα(37–46) are sufficient for nuclear export. An oligonucleotide specifying PKIα(37–46) was fused to the C-terminus of GST, and the distribution of GST–PKIα(37–46) was examined 30 min after nuclear injection.

(B) Residues 73–84 from HIV-1 Rev protein are also sufficient for nuclear export. Oligonucleotides specifying wild-type and mutant Rev(73–84) were fused to the C-terminus of GST. The mutated residues are underlined. The export abilities of wild-type and mutant GST–Rev(73–84) were examined 30 min after nuclear injection.

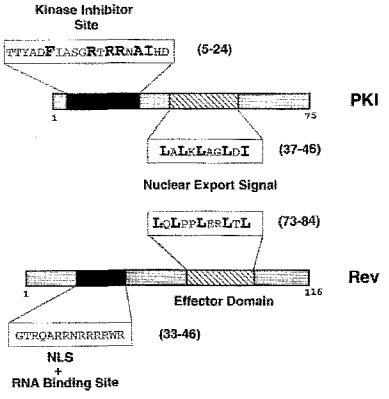

Figure 8. Comparison of Functional Domains between PKI and Rev

PKI has two separate and distinct functional domains, a kinase inhibitor site at the N-terminus and an NES near the C-terminus. HIV-1 Rev protein also contains two functional domains. The N-terminal domain is necessary and sufficient for RNA binding and nuclear localization. The C-teminal domain is necessary and sufficient for nuclear export. The functional domains from both PKI and Rev are boxed. Critical residues are in bold.

required for the export of pre-mRNAs that are essential for completion of the HIV-1 life cycle (Fischer et al., 1994). Mutations in the effector domain significantly impaired the export ability of this protein and result in a *trans*-dominant phenotype (Malim et al., 1989; Venkatesh and Chinnadurai, 1990). The sequence in this leucine-rich domain (LQLPPLERLTLD) is similar to the NES derived from PKI (LALKLAGLDIN), especially the last three leucines or isoleucines (underlined). Mutation of individual leucines to alanine prevented exit from the nucleus and destroyed biological activity (Meyer and Malim, 1994). The similarity of the sequences between NES of PKI and effector domain of Rev protein, as well as the capability of effector domain of Rev to export pre-mRNA from the nucleus, led us to test whether this effector domain is sufficient for nucleus export. To do this, the sequence from 73–84 of the Rev effector domain (LQLPPLERLTLD) was fused to GST (see Figure 7B). The fusion protein was then injected into the nucleus. The result revealed that this sequence is sufficient to direct the fusion protein from the nucleus to the cytoplasm. In contrast, a mutant form sequence (M10), with Leu-78 and Glu-79 replaced with Asp-78 and Leu-79, respectively, failed to export the fusion protein. This is consistent with the previous result that M10 is defective in exporting pre-mRNA from the nucleus. Therefore, in addition to PKI, Rev protein utilizes a similar hydrophobic motif to achieve the nuclear export of pre-mRNA.

Discussion

Compared with protein import, protein export from the nucleus has been poorly understood; this description of an NES that causes nontrafficking proteins to be exported from the nucleus is novel. This study has identified an NES, a short amino acid sequence in which hydrophobic residues are critical. This signal causes an ATP- and temperature-dependent extrusion from the nucleus that is

much more rapid and complete than previous examples of default shuttling of proteins bearing only an NLS (Schmidt-Zachmann et al., 1993; Laskey and Dingwall, 1993). In the latter case, most of the shuttling molecules are in the nucleus at any moment, but their occasional escape on a time scale of hours can be detected by sensitive trapping assays. By contrast, a protein displaying a functional NES is predominantly cytoplasmic at steady state and reaches that distribution within minutes after direct microinjection into the nucleus. Even proteins such as BSA or IgG, which are far too large to diffuse through nuclear pores, undergo net extrusion when conjugated to a functional NES (Borer et al., 1989; Goldfarb et al., 1986; Guiochon et al., 1991; Lanford et al., 1986). These results reconfirm that NES action cannot be explained by preferential binding to cytoplasmic rather than nuclear components, because such binding could only affect proteins that could cross the nuclear envelope.

Self-Sufficiency of the NES

Fusing the candidate NES to two unrelated proteins, GST and $R^1(\Delta 1–91)$, and also the chemical conjugations of synthetic NES peptide to BSA and IgG, was sufficient to induce export and exclusion from the nucleus. Moreover, fusing the NES to three different regions of GST gave similar results. These results suggest that the NES, like the NLS, is directly encoded by a short peptide rather than by a ternary structure generated by inducing changes in the conformation of the attached protein or juxtaposing other regions. The only case where the NES did not cause nuclear export and exclusion is native uncomplexed PKI itself, which readily enters the nucleus. The NES only appears to be exposed when the N-terminus of PKI binds to the C subunit (Fantozzi et al., 1994). Fusion of the N-terminus to GST or MBP is also sufficient to restore nuclear export (Wen et al., 1994). Because these three partner proteins share no structural similarity, the most likely explanation for their common action is that the NES is masked by the N-terminus of PKI until the latter is itself engaged in some other interaction (Wen et al., 1994). The C-terminus of native PKI may also assist in the masking of the NES, because fusion of the C-terminus to GST also activates export.

Sequence Specificity of the NES

Truncation analysis showed that the 15-mer PKIα(35–49) was a functional NES when fused to GST and $R^1(\Delta 1–91)$ or conjugated to BSA and IgG. Preliminary results showed that just the ten residues 37–46 are sufficient for nuclear export. Alanine-scanning mutagenesis showed that no individual hydrophilic residue was necessary, not even Lys-40, which is conserved between PKIα and PKIβ1. However, hydrophobic residues Ile-46, Leu-44, Leu-41, Leu-37, and possibly Leu-39 are required for a fully functional NES. Leu-37, Leu-41, and Leu-44 are known from solution NMR studies and secondary structure prediction to form a hydrophobic surface of a helix extending from residues 35 to 44 (A. Padilla et al., personal communication). Ile-46 lies just beyond the helix. These residues might fit together to form a hydrophobic surface recognized by the export apparatus. PKIβ1 has Val-46 instead of Ile-46, so at least one minor alteration seems acceptable as long as sufficient hydrophobicity is retained. These results also document the sequence specificity of the NES. Further studies will be necessary to determine whether other substitutions less drastic than alanine are tolerated at the key hydrophobic residues.

Do Any Other Molecules Use the Same NES or Export Machinery?

The sequence from the effector domain of HIV-1 Rev protein is also sufficient to export a GST fusion protein from the nucleus. Although the sequences in PKI (LALKLAGLDIN) and Rev (LQLPPLERLTLD) are not identical, they show great similarity. Both sequences are rich in hydrophobic residues, the last three of which are especially critical for nuclear export. However, the two underlined sequences alone are not sufficient for nuclear export (data not shown). The first two leucines might also contribute to the full nuclear export activity as they do in PKI. Another interesting molecule that appears to contain the NES-like sequence derived from PKI is IκBα (Haskill et al., 1991). However, it remains an open question whether the NES of PKI is representive of a general signal for nuclear export. It will be interesting, furthermore, to examine to what extent the pathway for PKI-mediated export and RNA export overlap.

Nuclear Export versus Nuclear Import

The protein containing both NES and NLS can transport in either direction and reaches an equilibrium, indicating that neither is overwhelming. Although the protein is slightly concentrated in the nucleus, suggesting that the NLS is relatively a little stronger than the NES, the final distribution may be determined by the rates of these two processes and could also be influenced by binding proteins in either compartment. Since the NLS is very basic, while the NES is very hydrophobic, these two motifs very likely target different sites to achieve translocation. On the other hand, both NLS- and NES-mediated processes are temperature and ATP dependent, and a similar mechanism may be involved in both processes. An important question to be addressed now is to what extent, if any, the machineries of nuclear import and export are shared.

Biological Significance

What is the biological purpose of a system for actively extruding RNA-free proteins from the nucleus? Proteins are synthesized in the cytoplasm, so if it is important for a particular protein to remain permanently cytoplasmic, a simpler strategy would be to keep it from entering the nucleus in the first instance, by increasing its size beyond the limit for passive diffusion, binding it to other cytoplasmic components, or avoiding NLSs. An NES would be most valuable when a protein has to be rapidly transported from the nucleus either to participate in a catalytic cycle or in response to changing conditions such as the cell cycle or modulations of external stimuli. In the former case, the NES would probably be combined with an NLS; in the latter, it should be possible to mask or unmask the NES

as required. While the constructs described in the present work provide examples of both, the full-length PKI expressed in cells provides an example of an NES that is only exposed when PKI is bound to C, and thus suggests a possible role for PKI in ejecting C subunit from the nucleus to ensure the cell cycle progression (Wen et al., 1995). Other examples of NES-containing peptides remain to be identified.

Experimental Procedures

Site-Directed Mutagenesis

Mutations in PKIα were introduced as described by Kunkel (1985). The *PKIα* cDNA was provided to us by Dr. R. Maurer (Thomas et al., 1991). All mutants were verified by sequencing and expressed in E. coli BL21(DE3) (Lysis).

Construction of Fusion Proteins

Construction of Deletion Fusion Proteins with GST

The N-terminal deletions of PKI (25–75, 35–75, and 44–75) were fused in-frame to pGEX-KG vector to generate GST–PKI fusion constructs. The C-terminal deletions of PKIα (1–56, 1–49, and 1–42) were fused to the N-terminus instead of the conventional C-terminus of GST to generate PKI–GST fusion constructs, because the latter fusions were consistently contaminated by small proteins, most likely owing to the proteolysis of PKI caused by C-terminal truncations. A vector, pRSETB-GST, which contains multiple cloning sites proceeding the N-terminus of GST, was constructed and used to this end.

Construction of Fusion Proteins Containing Short Sequences of PKI with GST

Synthetic oligonucleotides that specify short sequences of PKI were fused to either the N-terminus or the C-terminus of GST to generate H_6–PKIα(35–56)–GST, H_6–PKIα(35–49)–GST, H_6–PKIα(35–44)–GST, PKIα(35–49)–GST, GST–PKIα(35–49), and GST–PKIα(37–46) constucts.

Construction of Fusion Proteins with Regulatory Subunit (Type I, R^I)

Monomeric R^I subunit mutant cDNA ($R^I(\Delta 1–91)$) was introduced into the C-termini of H_6–PKIα(35–49) and H_6–PKIα(35–44) constructs to form H_6–PKIα(35–49)–$R^I(\Delta 1–91)$ and H_6–PKIα(35–44)–$R^I(\Delta 1–91)$.

Construction of Fusion Proteins Containing Both NLS and NES

A synthetic oligonucleotide corresponding to SV40 large T antigen NLS (PKKKRKVEDP) (Kalderon et al., 1984) was fused to the C-termini of H_6–PKIα(35–49)–GST and H_6–PKIα$(35–49)_{mut}$–GST, where Leu-41 and Leu-44 were replaced with alanines, to form H_6–NES–GST–NLS and H_6–PKIα$(35–49)_{mut}$–GST–NLS.

Construction of GST Fusion Proteins Containing Sequences of HIV-1 Rev Protein

Synthetic oligonucleotides specifying wild-type and mutant sequences of Rev(73–84) were fused to PGEX-KG to create GST–Rev(73–84) and GST–M10(73–84) constructs.

Preparation of Proteins

All fusion proteins containing a GST portion were purified on glutathione–agarose columns as described previously (Guan and Dixon, 1991; Harootunian et al., 1993; Wen et al., 1994). R^I fusion proteins were purified by metal (Ni^{2+}) chelate chromatography (Qiagen, Incorporated) as described by the manufacturer for polyhistidine fusion protein purification. Recombinant C subunit and PKI were expressed and purified as described previously (Wen and Taylor, 1994).

Fluorescence Labeling of Purified Protein

All the fusion proteins were labeled with FITC as described earlier and further purified on the Superose 12 column (Fantozzi et al., 1994; Wen and Taylor, 1994). All the complexes of mutant PKIs with FITC-labeled C subunit were purified on a Sephadex 75 column with a Pharmacia fast protein liquid chromatography instrument. All the labeled proteins were then concentrated to 60–100 μM.

Conjugation of Synthetic Peptides to Carrier Protein

Synthetic peptide P(35–49) encompassing PKI(35–49) (GSNELALKLAGLDINKTGGC-NH_2) and mutant peptide P$(35–49)_{mut}$ (GSNELALKAAGADINKGGC-NH_2), where Leu-41 and Leu-44 were replaced with alanines, were synthesized on a Milligen 9050 Pepsyn peptide synthesizer according to standard Fmoc methodology. Both peptides were purified by HPLC, and the sequences were confirmed by a gas phase amino acid sequencer with an on-line phenylthiohydantoine analyzer (Applied Biosystems, Incorporated, models 470A and 120). The synthetic peptides were conjugated to BSA and rabbit IgG with the bifunctional cross-linking reagent SMCC (Molecular Probes). SMCC in dimethylformamide was added to the carrier protein (1 mg/ml) in 10 mM potassium phosphate buffer (pH 7.0), with 25:1 molar ratio of SMCC and carrier protein. After incubation for 45 min at room temperature, the unreacted SMCC was removed by prepacked gel filtration column NAP-10 (Pharmacia) in the same buffer. The SMCC-coupled carrier proteins (1 mg/ml in the same buffer) were combined with peptides with 25:1 molar ratio of peptide and carrier protein and incubated for 3 hr at room temperature. The unconjugated peptide was removed by using a prepacked NAP-10 column. The conjugates of BSA (P(35–49)–BSA, P$(35–49)_{mut}$–BSA) were then labeled with FITC in 10 mM potassium phosphate buffer (pH 7.0). All the conjugations were analyzed by SDS–polyacrylamide gel to ensure a satisfactory coupling ratio.

Cells, Microinjection, and Fluorescence Imaging

Culture of REF52 cells, microinjection, and fluorescence imaging were performed under the same conditions as described previously (Fantozzi et al., 1994; Harootunian et al., 1993; Wen et al., 1994).

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