Three Leucine-Rich Sequences and the N-Terminal Region of Double-Stranded RNA-Activated Protein Kinase (PKR) Are Responsible for Its Cytoplasmic Localization[1]

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Received May 29, 2000; accepted June 23, 2000

The double-stranded RNA-activated-protein kinase PKR was originally identified as a ribosomal protein that regulates protein synthesis at the translational level. While PKR locates predominantly to the cytoplasm, nuclear or nucleolar species of PKR have been detected. Here, we demonstrate that PKR possesses three leucine-rich sequences resembling nuclear export signals (NESs). Enhanced green fluorescent protein (EGFP) fused to one of these sequences and transfected in COS-1 cells exhibited predominant cytoplasmic staining, which was abrogated by a leucine to alanine substitution. In addition, Leptomycin B (LMB), an inhibitor of NES-mediated nuclear export, inhibited the cytoplasmic localization of EGFP-NES, indicating the potential activity of these stretches as NESs. Although EGFP fused to a PKR with three NES mutations still located to the cytoplasm, an additional N-terminal deletion impaired the cytoplasmic predominance, suggesting that the N-terminal region is also required for localization. These results suggest that the cytoplasmic localization of PKR is regulated by NESs as well as the N-terminal sequence.

Key words: EGFP, Leptomycin B, NES, NLS, PKR.

The interferon (IFN)-inducible, double-stranded RNA–activated protein kinase (PKR) is a serine/threonine kinase ubiquitously expressed in mammalian cells (*1, 2*). PKR is activated *via* double-stranded RNA molecules generated by viral infection or RNAs with secondary stem loop structures (*3*). Upon activation, PKR autophosphorylates, after which it phosphorylates eukaryotic translational initiation factor 2 (eIF-2α) (*4*), resulting in an inhibition of protein synthesis at the initiation level, and consequent blockage of cell growth or viral replication (*5*). Thus, PKR has been considered to participate in the host defense mechanism induced by type I IFNs (*5*). In addition, PKR overexpression has been shown to inhibit cell growth (*6*) and induce apoptosis (*7–9*). In contrast, the expression of a catalytically inactive mutant of PKR transforms NIH-3T3 cells (*10–12*). This accumulating evidence indicates crucial roles for PKR in cell growth, differentiation and the induction of apoptosis.

PKR is a cytoplasmic enzyme that has been shown to be associated with the rough endoplasmic reticulum (*13*). This observation is consistent with the identification of its binding to ribosome protein L18 (*14*). PKR is also observed in the nucleus, especially in the nucleolus (*15*). Nuclear species of PKR have been suggested to play a role in ribosomal synthesis (*16*), or to interact with nuclear RNA species (*17*). Although the distribution of PKR between the cytoplasm and nucleus has been discussed in terms of its post-translational modification (*15*), the functional role of the nuclear PKR is largely unknown. On the other hand, PKR-like eIF2-α kinase exists in the endoplasmic reticulum where it mediates stress response (*18, 19*). This evidence further implies that the subcellular localization of PKR plays some role in its signaling.

The nuclear localization of proteins is mediated by nuclear localization signals (NLSs) that are usually composed of a short stretch of positively charged amino acids (*20*). In contrast, leucine-rich nuclear export signals (NESs) play a crucial role in the export of proteins from the nucleus (*21, 22*), and several factors have been found to be involved in this pathway (*23*). One of these factors, CRM1, has been shown to be a receptor for the leucine-rich NES (*23*). Moreover, a potent antifungal antibiotic, leptomycin B (LMB), inhibits NES-mediated nuclear export by binding directly to CRM1 (*24, 25*).

[1] This work was supported by Grants-in-Aids from the Ministry of Education, Science, Sports, and Culture of Japan, Aichi Cancer Research Foundation, Special Coordination Fund for Promoting Science and Technology, and funds from the Terumo Life Science Foundation.

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Abbreviations: EGFP, enhanced jellyfish green fluorescent protein; eIF, eukaryotic translational initiation factor; FITC, fluorescein isothiocyanate; IFN, interferon; LMB, leptomycin B; NES, nuclear export signals; NLS, nuclear localization signal.

In this paper, we present evidence for NESs in human PKR, and suggest that these NESs play a role in the cytoplasmic localization of PKR in cooperation with its N-terminal amino acid sequence.

MATERIALS AND METHODS

Cell Lines and DNA Transfection—COS-1 and human embryonic kidney 293 (293) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, and maintained under 5% CO_2 at 37°C. Cells seeded on cover glasses were transfected with 2 μg of plasmid DNA per 5 × 10^6 cells using 6 μl of Lipofectamine-plus and 4 μl of Lipofectamine (GibcoBRL, MD, USA) according to the manufacturer's instructions. Subsequently, cells were cultured in the presence or absence of LMB at a concentration of 1 ng/ml. For treatment of cells with poly(I)-poly(C), 293 cells were cultured in the presence or absence of poly(I)-poly(C) (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at a concentration of 100 μg/ml for the indicated periods. Immunoblot analysis was performed as described previously (*9*) using an anti-PKR polyclonal antibody (N-18, Santa Cruz, CA, USA) or anti-GFP polyclonal antibody (Santa Cruz).

Indirect Immunofluorescence—The day after transfection, cells were fixed in 4% paraformaldehyde in PBS for 30 min, and the plasma membranes were permeabilized with 0.2% Triton X-100 for 5 min at room temperature and washed with PBS. Subsequently, the treated-cells were incubated with anti-PKR polyclonal antibody (N-18) for 60 min. The cells were then stained with 100-hold diluted fluorescein isothiocyanate (FITC)–labeled anti-rabbit antiserum (MBL, Nagoya) for 60 min, and observed under a fluorescence microscope at 400× (Olympus BX-60, Tokyo). For the expression of enhanced jellyfish green fluorescent protein (EGFP) (Clontech Laboratories, Palo Alto, USA), the cells were fixed in 4% paraformaldehyde, and observed under a fluorescence microscope as described above.

Plasmid Constructs—Human PKR cDNA subcloned into pBluescript vector (Stratagene, La Jolla, USA) was kindly provided by Dr. Ara Hovanessian (Institut Pasteur, France). A PKR cDNA carrying a lysine to arginine point mutation at position 296 was constructed as previously described (*26*). Construction of deletion mutants of EGFP-PKR in Fig. 3 was as follows. pEGFP-PKR K/R was digested with *Eco*RI, and self-ligated to generate clone No. 2. pEGFP-PKR K/R was digested with *Bam*HI, and partially digested with *Bgl*II, after which the resulting DNA fragments were self-ligated in order to generate clones No. 3 and 4. pBluescript-PKR was digested with *Bgl*II and *Pst*I, after which the *Bgl*II–*Pst*I fragment was subcloned into the *Bam*HI/*Pst*I sites of pEGFP-C3 to generate clone No. 5. pEGFP-PKR K/R was digested with *Sca*I and *Acc*I, and the site blunt-ended by Klenow enzyme and self-ligated to generate clone No. 6. Clone No. 7 was generated by *Bgl*II digestion of pBluescript-PKR, which was subcloned into the *Bam*HI site of pBluescript. Subsequently, the plasmid was digested with *Pst*I and *Xba*I, and the resulting fragment was subcloned into the *Pst*I/*Xba*I sites of pEGFP-C3.

DNA fragments containing one of the three NES sequences (I, II, III) in Fig. 4A were PCR-amplified using 5′ primers, 5′-ATCTCGAGGGTTCTACTAAACAGGAA-3′ for NES I (NES If), 5′-ATCTCGAGCAATGGATTGAAAAAAGAAGA-3′ for NES II (NES IIf), 5′-ATCTCGAGTCTTCGCAAGACTATGGA-3′ for NESIII (NES IIIf), and 3′ primers, 5′-CAGAATTCGGTAGTCAGATTTCACTGA-3′ for NES I (NES Ir), 5′-CAGAATTCGTATATAATCCACCCCTTT-3′ for NES II (NES IIr), and 5′-CAGAATTCGTTCAAAAGCAGTGTCACA-3′ for NES III (NES IIIr). All of these primers produced 90-bp DNA fragments with *Xho*I and *Eco*RI sites at their 5′ and 3′ ends, respectively. Each fragment was digested with *Xho*I and *Eco*RI, and subcloned into the *Xho*I/*Eco*RI sites of pEGFP-C3. Mutagenesis of L to A in each NES sequence (underlined in Fig. 2A) was performed by sequential PCR steps (*27*). For the construction of mutant NES I (NES Im), the 5′ half of NES I was amplified using NES If as a 5′ primer and 3′ primer, 5′-AGCATATGCAGCTTTAGCGGCCAAT-3′ (NES Imr); the 3′ half was amplified using 5′ primer, 5′-AGCTGCATATGCTCAGATATTATCA-3′ (NES Imf) as well as NES Ir as a 3′ primer. The resulting two DNA fragments were annealed, and then a second PCR was caried out using NES If and NES Ir. The amplified product was digested with *Xho*I and *Eco*RI, and subcloned into the *Xho*I/*Eco*RI sites of pEGFP-C3. For the construction of NES IIm and NES IIIm, the same strategy was employed using NES IImf (5′-GTTGCGGCTGCGGAACTCTTTGAA-3′), NES IImr (5′-CGCAGCCGCAACTTTGTCTAGT-3′), NES IIImf (5′-TGCGGGGGCAATTGCTGCTGAACTTCTT-3′), and NES IIImr (5′-ATTGCCCCCGCAGCGTAGAGGTCC-3′) primers. The amplified DNA fragments with mutations were verified by DNA sequencing using the dideoxy chain termination method (*28*).

pEGFP-PKR K/R, containing mutants NES I-III, was constructed by the sequential PCR method described above. A DNA fragment upstream of the mutant NES I was amplified using 5′ primer, 5′-CCGTGATTATCTGCGTGCAT-3′ (PKR/up) and the 3′ primer of NES Imr. A downstream fragment was amplified with the 5′ primer of NES Imf and 3′ primer, 5′-TCACAGAATTCCATTTGGAT-3′ (PKR-4). The resulting two DNA fragments were then annealed, and secondary PCR was performed using PKR/up and PKR-4 primers. For the construction of DNA fragments containing NES IIm and IIIm, the same strategy was employed. The downstream fragment of mutant NES III was PCR-amplified using the 3′ primer of 5′-CTGTTTCTGCAGAAAGATTAGTAAAAATAG-3′ (PKR-Pst). The primers used to generate these fragments are outlined in Fig. 2B. All the fragments obtained containing mutations in NES I, II, or III were digested with *Hin*dIII/*Eco*RI, *Eco*RI/*Bgl*II, or *Bgl*II/*Pst*I, respectively (Fig. 2B), ligated and subcloned into *Hin*dIII/*Pst*I-digested pEGFP-C3. The amplified DNA fragments with mutations were verified by DNA sequencing as described.

RESULTS AND DISCUSSION

Localization of PKR and EGFP-PKR—Human PKR or mutant PKR without kinase activity were transfected into COS-1 cells and their expression or localization was examined by Western blotting or immunostaining using anti-PKR or anti-GFP antibody, respectively. While wild type PKR was poorly expressed because of the generalized inhibition of protein synthesis, a significant amount of mutant PKR was produced (Fig. 1A, lanes 1 to 3), as previousely reported (*29*). The wild-type and mutant PKR showed predominant cytoplasmic localizations (Fig. 1B, 1 to 3). Consistently, about 80% of total PKR has been shown to be present in the cytoplasm while 20% is in the nucleus (*15*). We recently constructed EGFP-PKR fusion proteins to visualize the direct effect of PKR on cells (*9*). The expression of EGFP-PKR was very poor, while that of EGFP-mutant PKR was significant (Fig. 1A, lanes 4 to 6), with a pattern almost the same as that of PKR without EGFP as described above. Both EGFP-PKR and EGFP-mutant PKR localized mainly in the cytoplasm, whereas EGFP was distributed diffusely in the cells (Fig. 1B, 4 to 6). These results suggest that EGFP does not significantly affect the cytoplasmic localization of PKR. In addition, the nucleolar staining of EGFP-PKR K/R was also remarkable (Fig. 1B, No. 6), indicating that EGFP-PKR K/R is capable of entering into the nucleus. Since the appearance of a report indicating that EGFP-PKR alone causes apoptosis (*9*), we employed EGFP fusion proteins derived from the inactive kinase mutant of PKR for our experiments.

Nuclear Export Activity in NESI, II, and III—We found three leucine-rich sequences (residues 156–166; 386–394; 471–482) in PKR that resemble the NES sequences of PKI or Rev (*21*, *22*) (Fig. 2A). These sequences were tentatively named NES I, NES II, and NES III from the N-terminus of PKR. To elucidate the role of these NES-like sequences in the subcellular localization of PKR, we first examined the expression of EGFP fused to a series of PKR deletion mutants (Fig. 3A) and transfected into COS-1 cells. These constructs were expressed effectively in COS-1 cells, as examined by Western blotting (Fig. 3B), except No. 5, containing NES III, the expression of which was less effective than the other clones. The mutant in which all NES sequences were deleted (No. 2 in Fig. 3C) was diffusely distributed in COS-1 cells, while mutants containing NES I alone (No. 3), NES I and II (No. 4), or NES III alone (No. 5) localized mainly in the cytoplasm, suggesting that NES I or III is sufficient for

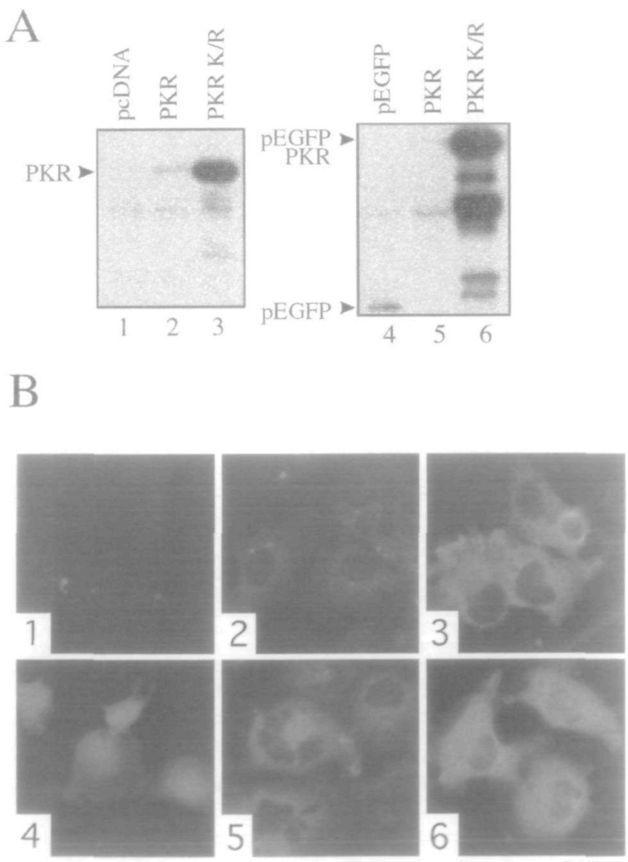

Fig. 1. **The expression and subcellular localization of PKR, PKR K/R, EGFP-PKR, and EGFP-PKR K/R.** (A) COS-1 cells were transfected with 2 μg of each plasmid as indicated above the lanes. Cells were lysed in lysis buffer 30 h after transfection. The lysates were then resolved by SDS-PAGE and transferred to Immobilon-P membranes. The blots were probed with anti-PKR polyclonal antibody (lanes 1 to 3) or anti-GFP polyclonal antibody (lanes 4 to 6), followed by anti-rabbit immunoglobulin antibody labeled with horseradish peroxidase. Signals were visualized by ECL. (B) COS-1 cells were transfected as described in (A) and fixed 18 h after transfection. The cells were then processed for indirect immunofluorescence using rabbit anti-PKR antibody followed by FITC-conjugated anti-rabbit antiserum (1 to 3) or observed directly (4 to 6). The localization of PKR was determined under a fluorescence microscope at 400×. Exposure time was about 3 min under these conditions for 1, 2, and 5, and about 5 s for 3, 4, and 6. Numbers correspond to the lanes in (A).

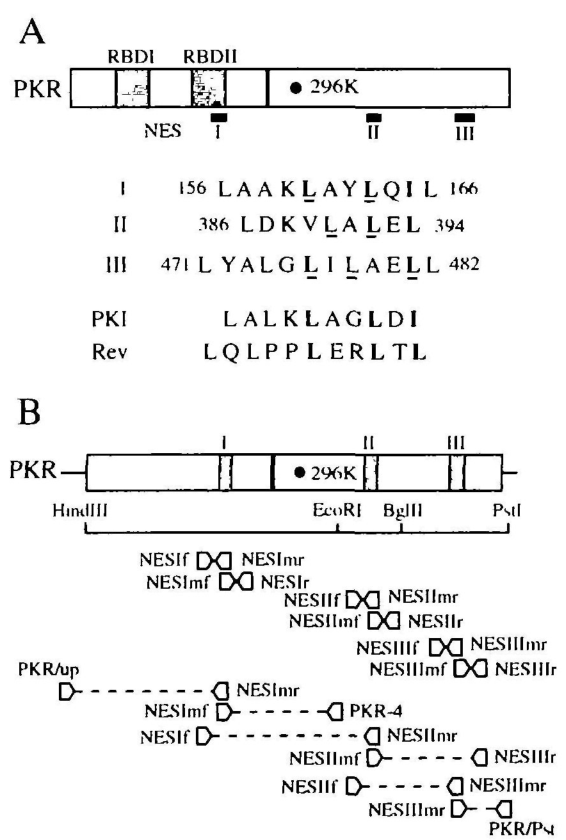

Fig. 2. **Leucine-rich NES-like sequences in PKR.** (A) Schematic representation of the domain structure of PKR. The vertical thick line divides PKR into two portions, a regulatory domain on the left and a catalytic domain on the right. The positions of the three leucine-rich sequences are indicated by thick short lines as NES I, II, III. RBDs denote double-stranded RNA binding domains. 296K represents a lysine to arginine substitution in the inactive kinase mutant of PKR K/R. The amino acids of NES I, II, and III in PKR were compared with NESs of PKI and Rev. The most conserved leucine and isoleucine residues are shown in bold, and leucine to alanine substituted residues in Figs. 4 and 5 are underlined. (B) Construction of PKR containing mutants NES I–III by sequential PCR. The oligonucleotide primers used to generate mutations are shown by pentagonal boxes. Primer sequences are described in "MATERIALS AND METHODS."

the cytoplasmic localization of EGFP. However, deletion mutants containing either NES II alone (No. 7), or NES II and III (No. 6) showed no preferential cytoplasmic localization. These results suggest that such NES sequences do not contribute equally to the localization of PKR, and that the structure of PKR may affect NES function.

To examine the nuclear export activity of these NESs, EGFP was fused to a 30–amino acid sequence containing one of these NESs with neighboring amino acids (Fig. 4A), and transfected into COS-1 cells. The expression levels of these constructs were almost the same as determined by Western blotting (Fig. 4B). The EGFP fused to each NES located mainly in the cytoplasm, whereas control EGFP-C33, which contains 33 amino acids derived from multicloning sites of pEGFP and pBluescript, was found diffusely distributed in COS-1 cells (Fig. 4C). In contrast, the expression of EGFP fused to one of the mutant NESs containing leucine to alanine substitutions (Fig. 2A, under-

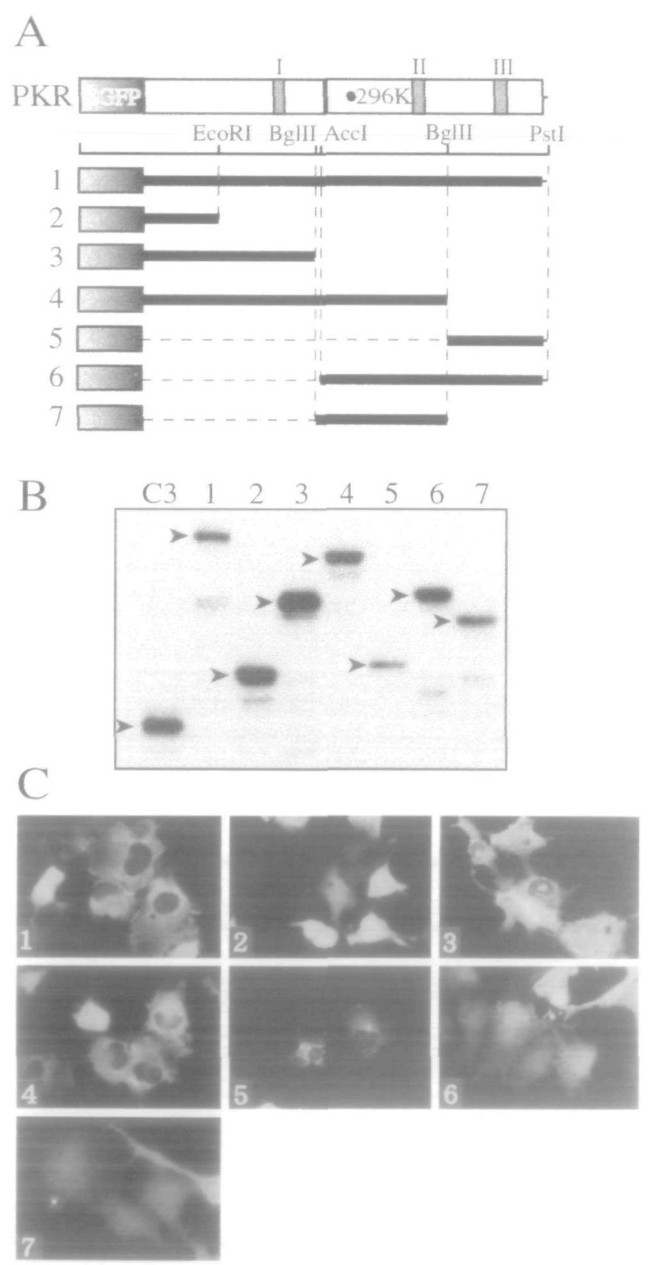

Fig. 3. **Effect of a series of PKR deletions on the subcellular localization of EGFP-PKR K/R.** (A) Diagram of EGFP-PKR K/R deletion mutants. Restriction sites used to generate deletion mutants are indicated. NES I, II, III are boxed. The gradient box denotes EGFP. PKR fragments connected to EGFP are depicted by thick lines. (B) The expression of the deletion mutants was examined by immunoblotting using anti-GFP antibody as described in Fig. 1A. Arrowheads indicate the corresponding signals of each construct. C3 denotes the empty vector of pEGFP-C3. (C) Localization of EGFP-PKR K/R deletion mutants. COS-1 cells were transfected with each construct, and the location of EGFP-fusion was determined 18 h after transfection by fluorescence microscopy at 400×. Numbers correspond to those of the construct in (A).

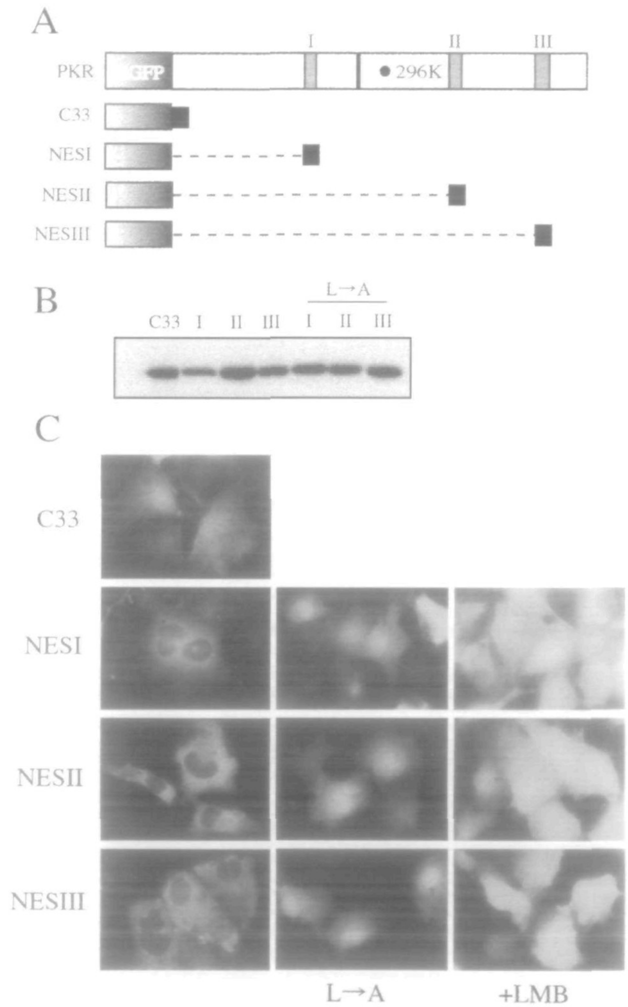

Fig. 4. **Subcellular localization of EGFP-NES.** (A) Diagram of EGFP-NES I, II, and III. C33 denotes EGFP fused to the control 33–amino acid sequence derived from multicloning sites of pEGFP and pBluescript. Each NES sequence contains a total of 30 amino acids with leucine-rich residues indicated in Fig. 2A, and neighboring amino acids. (B) Expression of each construct was examined by immunoblotting using anti-GFP antibody as described for Fig. 1A (C). Subcellular localization of EGFP-NESs. COS-1 cells were transfected with each construct, and 18 h post-transfection the location of EGFP-NES was determined by fluorescence microscopy at 400×. L→A indicates COS-1 cells transfected with mutant NESs (the alanine substituted for the leucine residue is underlined in Fig. 2A). +LMB indicates COS-1 cells that were incubated in the presence of LMB at 1 ng/ml for a further 2 h after transfection.

lined), lost its preferential cytoplasmic localization (Fig. 4C, L→A). This result is consistent with the previous report that leucine residues in NESs are important for its activity (*21*). To confirm NES activity further, we examined the effect of LMB, which has been shown to inhibit nuclear export by binding directly to an NES receptor, CRM1 (*25*). When COS-1 cells expressing the EGFP fused to one of the NESs, were exposed to 1 ng/ml LMB for 2 h, the predominant cytoplasmic localization of EGFP-NES was abrogated (Fig. 4C, +LMB). These results strongly support the notion that all NES sequences in PKR posses nuclear export activity.

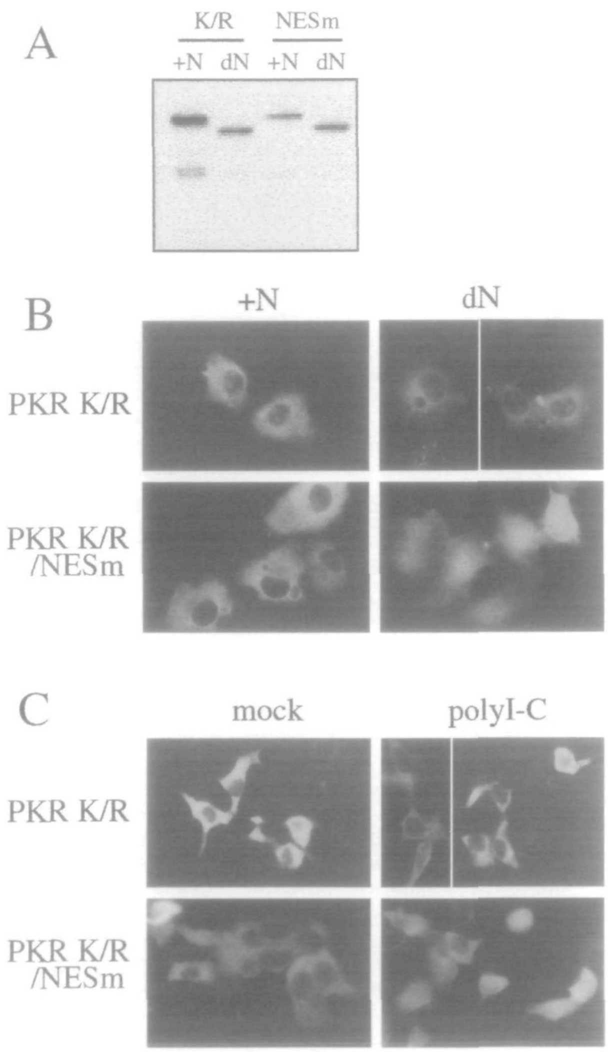

Fig. 5. **Subcellular localization of EGFP-PKR K/R with all the mutated NESs (EGFP-PKR K/R/NESm).** The effect of an N-terminal 97-amino acid deletion on the location of EGFP-PKR K/R/NESm. COS-1 cells were transfected with pEGFP-PKR K/R (PKR K/R) or pEGFP-PKR K/R/NESm (PKR K/R/NESm) without N-terminal deletion (+N) or with deletion (dN). (A) The expression of each construct was examined by immunoblotting using anti-GFP antibody as described. (B) The location of each EGFP-fusion protein was determined 18 h post-transfection under a fluorescence microscope at 400×. (C) The effect of poly(I)-poly(C) on the location of EGFP-PKR K/R/NESm. 293 cells were transfected with pEGFP-PKR K/R (PKR K/R) or pEGFP-PKR K/R/NESm (PKR K/R/NESm), and 18 h post-transfection, the cells were incubated in the absence (mock) or presence (polyI-C) of poly(I)-poly(C) at 100 μg/ml for a further 6 h. Subsequently, the location of EGFP-fusion protein was determined as in (B).

Other Factors Required for the Cytoplasmic Localization of PKR—EGFP-PKR K/R with all mutant NESs (PKR K/R/NESm) was found to be located predominantly in the cytoplasm (Fig. 5B, lower left), suggesting that factor(s) other than NESs are required for the cytoplasmic localization of PKR. To exclude the possibility that the EGFP-tag inhibits PKR entry into the nucleus, we examined transfection with pcDNA-PKR K/R/NESm, which does not contain EGFP, and found that PKR K/R/NESm still showed the cytoplasmic localization (data not shown). Thus, the EGFP was not responsible for the localization. We next examined the effect of N-terminal deletion in addition to NES mutations on PKR localization. The expression of these mutants was verified by Western blotting using anti-GFP antibody (Fig. 5A). Transfection of COS-1 cells with pEGFP-PKR K/R/NESm lacking the N-terminal 97 amino acids (dN) showed a diffused distribution of fluorescence (Fig. 5B, lower right), whereas PKR K/R (dN) without NES mutations was localized to the cytoplasm (Fig. 5B, upper right). Thus, the N-terminal region of PKR is also required for the cytoplasmic localization. Since the N-terminal region has been shown to interact with dsRNA, the effect of poly(I)-poly(C) on the subcellular localization was examined. We previously observed that poly(I)-poly(C) exposure increases Fas expression (*30*). Substantial amounts of EGFP-PKR K/R/NESm change its localization to both nucleus and cytoplasm upon poly(I)-poly(C) exposure, whereas EGFP-PKR K/R does not (Fig. 5C). These results suggest that not only NESs, but also the N-terminal region of PKR, both determine subcellular localization. Since some proteins, such as ribosomal protein L18, have been shown to interact with the N-terminal region of PKR (*31*), PKR may remain in the cytoplasm associated with protein(s) *via* the N-terminal region. As dsRNA reportedly competes with L18 for binding to the N-terminal region, PKR may enter the nucleus when released from interacting proteins by dsRNA. Whether PKR enters the nucleus with or without dsRNA, and the involvement of other PKR regions in its localization, especially the third basic region to which $P58^{IPK}$ binds (*32*), remain to be investigated.

Several possible functions of nuclear PKR have been suggested, such as involvement in ribosome biosynthesis (*16*), and interaction with nuclear RNA species, for instance Alu (*17*). NESs in PKR might signify a role in the export of nuclear RNA species. Alternatively, nuclear PKR may interact with p53 as reported *in vitro* (*33*), and might be so toxic as to necessitate its immediate export from the nucleus. Since the molecular weight of PKR seems to be too large for diffusion into the nucleus, it is reasonable to assume that PKR possesses an active nuclear import mechanism. Similarly, putative nucleophilic sequences have been suggested, although these have not yet been characterized (*15*). The NES mutants constructed in this study may unravel the question of NLS function, and facilitate further the understanding of the role(s) and mechanism(s) of nuclear import and export of PKR.

We thank Dr. Ara Hovanessian for kindly providing the PKR cDNA.

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