Intracellular Localization of the Ret Finger Protein Depends on a Functional Nuclear Export Signal and Protein Kinase C Activation*

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The Ret finger protein (RFP) was identified initially as an oncogene product and belongs to a family of proteins that contain a tripartite motif consisting of a RING finger, a B box, and a coiled-coil domain. RFP represses transcription by interacting with Enhancer of Polycomb and is localized to the cytoplasm or nucleus depending on the cell type. Here, we have identified the nuclear export signal (NES) located in the coiled-coil region of RFP. Mutation of this NES or treatment with leptomycin B abrogated the nuclear export of RFP in NIH3T3 cells. In addition, fusion of this NES to other nuclear proteins, such as yeast transcription factor Gal4, resulted in their release into the cytoplasm of NIH3T3 cells. Although the NES function of RFP in HepG2 cells is masked by another domain in RFP or by another protein, 12-O-tetradecanoylphorbol-13-acetate treatment or overexpression of constitutively active protein kinase C (PKCα) abrogated masking, leading to the cytoplasmic localization of RFP. Furthermore, treatment of NIH3T3 cells with PKC inhibitors blocked the function of NES, resulting in nuclear localization of RFP. Thus, the nuclear export of RFP is regulated positively by PKC activation. However, RFP was not a direct substrate of PKC, and additional signaling pathways may be involved in the regulation of nuclear export of RFP.

To fulfill their functions in complex organisms, most proteins possess specialized domains that have been conserved throughout evolution. Depending on their overall domain structure, proteins have been grouped into gene families, whose members often share similar functions in cells or organisms. Members of one such gene family, the RING-B box-coiled-coil (RBCC) family (1), are characterized by their possession of a tripartite motif consisting of a RING finger (2, 3), one or two B boxes (4), and an α-helical coiled-coil domain (5). These domains are involved in protein-protein interactions and allow RBCC family members to participate in various cellular processes depending on their subcellular localization. In transformed cells, three members of the RBCC family (PML, transcriptional intermediary factor 1α, and RFP) were found to be oncogenic after their RBCC domains were linked to other proteins by DNA rearrangements. In acute promyelocytic leukemia cells, a reciprocal chromosomal translocation fuses the PML gene with the retinoic acid receptor α gene, resulting in the expression of a PML-RARα fusion protein (6, 7) that blocks hematopoiesis at the promyelocytic stage (8, 9). Similarly, the N-terminal RBCC domain of transcriptional intermediary factor 1α recombines with the serine/threonine kinase domain of B-Raf in the T18 oncogene (10), and the N terminus of the Ret finger protein (RFP) was found to be fused to the ret proto-oncogene (11) in transformed NIH3T3 cells (12, 13). The RBCC motifs are required for the transforming capacities of these oncogenes (14), and oncogenesis may depend on the distinct cellular localizations of the fusion proteins (9, 15).

After the identification of the oncogenic RFP-Ret fusion protein in NIH3T3 cells, RFP was cloned from human (13) and mouse (16) and found to be highly expressed in pachytenic spermatocytes and round spermatids during spermatogenesis, suggesting that it participates in male germ cell differentiation (16, 17). Depending on the cell type or tissue, RFP is localized either to the cytoplasm or nucleus (16, 17). This localization requires the RBCC motif and homodimerization through the coiled-coil domain (18). In the nucleus, RFP associates with the nuclear matrix (19) and is a component of PML nuclear bodies (20) where it binds directly to PML (21). Furthermore, RFP can interact with the int-6 gene product, another component of PML nuclear bodies (22). Recently, RFP was shown to repress transcription by interacting with Enhancer of Polycomb (23), a member of Polycomb proteins. The Polycomb group proteins were originally identified in Drosophila as being involved in the maintenance of the correct expression pattern of homeotic genes. Polycomb group proteins form a large macromolecular complex and are involved in the epigenetic gene silencing. However, no biological function has yet been defined for RFP in the nucleus or cytoplasm, and mechanisms governing RFP subcellular localization remain largely unknown.

The separation of the cytoplasm and nucleus in eukaryotic cells provides an important way to regulate cellular processes through compartmentalization. As was shown for NF-AT (24) or IκBα (25, 26), nuclear import and export through the nuclear envelope can control gene expression in a signal-dependent manner by restricting the access of transcription factors to their target genes. The exchange between cytoplasm and nucleus occurs through the nuclear pore complex, and this process is mediated in most cases by specific amino acid sequences. Localization sequences exist for nuclear localization (nuclear localization sequence, NLS) (27, 28) and for nuclear export (nuclear export signal, NES) (29–31). The leucine-rich NES is
specifically recognized by CRM1, which functions as an export receptor mediating the fast release of proteins with this sequence from the nucleus (32–34). Masking of the NLS or NES through their binding to other factors or through post-translational modifications would allow for controlled shuttling of factors between the cytoplasm and the nucleus. Here we have studied the cellular localization of RFP and found that it can shuttle between the cytoplasm and the nucleus. Nuclear export of RFP is dependent on a functional NES, which can be activated by protein kinase C (PKC). The regulation of RFP localization by PKC points to an important role for RFP in the control of cellular differentiation and proliferation.

MATERIALS AND METHODS

Cell Culture—The NIH3T3 and Hep2G cells were maintained at 37°C in a 5% CO₂-containing atmosphere using Dulbecco’s modified Eagle’s medium (Nisus Pharmaceuticals) with 10% bovine calf serum (HyClone) or 10% fetal calf serum (Roche), respectively. 12-O-Tetradecanoylphorbol-13-acetate (TPA), H7, and staurosporine were obtained from Sigma. Leptomycin B (LMB) was a gift from Dr. M. Yoshida. Drug concentrations and incubation times are indicated in the figure legends. Serum concentrations were reduced to 2% when drugs were added to the cell cultures.

Expression Vectors—FLAG-tagged forms of RFP were expressed from pSG5-FLAG-Nt, and Gal4 fusion proteins from pCMX-Gal4 and pG4polyII (35). For the expression of GFP fusion proteins, the vectors pEGFP-C2, pEGFP-C3, pEGFP-N3, and pEFYP-Nuc (CLONTECH) were used. Mutations in the putative NES and PKC phosphorylation sites were introduced by polymerase chain reaction and verified by DNA sequencing. Expression vectors for PKCα were described by Ueda et al. (36). To express the activated Ha-Ras, the SV40 promoter-containing vector pCEV-1 was used. The SBo promoter-containing vector was used to express Jun N-terminal protein kinase 1 (JNK1).

Immunohistochemistry—Subcellular localization of RFP and colocalization with other proteins were determined as described by Khan et al. (37). For each 3-cm dish, 2 × 10⁴ NIH3T3 cells or 4 × 10⁵ Hep2G cells were seeded, and on the next day the cells were transfected with 3 or 1.5 μg of the expression vector using LipofectAMINE (Life Technologies, Inc.). 48 h after transfection, cells were fixed and stained with mouse monoclonal anti-FLAG antibody M2 (Sigma) and rabbit polyclonal antibodies against PKCα (Santa Cruz). Fusion proteins containing the DNA binding domain of Gal4 (Gal4-DBD) were detected by mouse monoclonal anti-Gal4-DBD antibody (Santa Cruz). The signals were visualized by rhodamine- or fluorescein isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch), and analyzed by confocal microscopy (Zeiss LSM510). Nuclear DNA was stained with TO-PRO®-3 iodide (Molecular Probes) and used as a nuclear marker. GFP fusion proteins were analyzed in the same way directly after fixation and DNA staining with TO-PRO®-3 iodide. In Figs. 7–9, two types of staining pattern, which showed the predominantly nuclear or cytoplasmic RFP, were observed, and the cells showing uniform distribution in both nucleus and cytoplasm were not detected. Approximately 50–150 cells were examined, and the percentages of cells with cytoplasmic RFP were calculated. Photographs and numbers from representative experiments are shown in the figures.

Yeast Two-hybrid Assay—The homodimerization of RFP was studied by the LexA yeast two-hybrid system in the strain L40 (38) using the vectors pBTM116 (38) for the expression of LexA fusion proteins and pASV3 (39) for the expression of VP16 fusion proteins. β-Galactosidase assays on individual transformants were carried out as described in Ref. 40.

Western Blotting and PKC Activity—PKC activity in NIH3T3 and Hep2G cells was measured in crude extracts using a PKC assay kit (Calbiochem). Detection of PKCα and a phosphorylated form of PKCα/β (Thr-638) was performed by Western blotting using 50 μg of total protein from NIH3T3 and Hep2G cells, anti-PKCα (C-20, Santa Cruz), or an anti-phospho-PKCα/β (Thr-638) antibody (New England Biolabs).

RESULTS

A Specific Domain of RFP Is Required for Its Cytoplasmic Localization in NIH3T3 Cells—RFP is highly expressed in tumor cell lines, and its subcellular localization depends on cell type. Exogenously expressed mouse RFP is located predomin-antly in the cytoplasm of NIH3T3 cells, whereas it is localized in the nucleus of Hep2G cells (18). We therefore chose NIH3T3 and Hep2G cells to study mechanisms that control human RFP shuttling between the nucleus and cytoplasm. Expression vectors for FLAG-tagged RFP (Fig. 1A) or an enhanced green fluorescent protein (EGFP)-labeled form of RFP were transfected into NIH3T3 and Hep2G cells, and their localization was analyzed by confocal microscopy using TO-PRO®-3 iodide as a nuclear marker. As expected, both forms of human RFP were found in the cytoplasm of NIH3T3 cells (Fig. 1C), associated with structures that resembled the endoplasmic reticulum or Golgi apparatus. Depending on the expression level, RFP showed a grainy pattern or clear dot-like structures. Small amounts of RFP were also visible in the nucleus, where they appeared as small dots. In contrast to NIH3T3 cells, both forms of human RFP localized to the nucleus of Hep2G cells (Fig. 1C). Staining was observed for the whole nucleoplasm with some preferences for the perinuclear region. Although RFP was reported to associate with PML nuclear bodies (21), colocalization...
of RFP and PML nuclear bodies was observed only at low frequency.

We next analyzed the mechanisms controlling the cytoplasmic localization of RFP in NIH3T3 cells. Because N-terminally linked EGFP-RFP fusion proteins showed a localization similar to that of FLAG-tagged RFP in both NIH3T3 and HepG2 cell lines (Fig. 1), EGFP-labeled deletion mutants of RFP were used to determine which domains of RFP affected its localization in NIH3T3 cells. Expression vectors encoding EGFP alone or EGFP fusion proteins for N-terminal and C-terminal deletion mutations of RFP were transfected into NIH3T3 cells, and their subcellular localization was analyzed along with a nuclear marker (Fig. 2). The EGFP control protein was localized in both the nucleus and cytoplasm because its small molecular mass of 27 kDa allows it to diffuse freely into the nucleus. However, the RFP fusion proteins used in this study had molecular masses in excess of 40 kDa, which may preclude their passive diffusion into the nucleus (30). Although the deletion of the RING finger domain of RFP (RFP96–513) had little effect on RFP localization, further deletion of the RING finger and B box, as in EGFP-RFP(132–513), led to distinct patterns of localization. Although the dot-like structures in the cytoplasm were maintained, increased staining of the nucleoplasm was observed. An additional deletion of the coiled-coil region (EGFP-RFP(315–513)) resulted in the disappearance of the dot-like structures in the cytoplasm and redistribution of RFP throughout the whole cell with a preference for the nucleus. A similar pattern was observed for a mutant lacking the C-terminal part of RFP, which contains the RING finger and the B box (EGFP-RFP(1–132)), although the distribution between the cytoplasm and nucleus was more uniform. Addition of the coiled-coil region to the RING finger and the B box (EGFP-RFP(1–258)) reestablished the localization of RFP as dot-like structures in the cytoplasm. Because the deletion analysis of RFP pointed to an overall importance of the coiled-coil region for RFP localization, a deletion mutant of RFP (EGFP-RFPΔcc), lacking the coiled-coil region, was constructed, and its subcellular localization was analyzed. As expected, the protein was distributed throughout the whole cell with preferential localization to the nucleus. These results indicate that the coiled-coil region is required for the cytoplasmic localization of RFP in NIH3T3 cells.
Identification of the NES in RFP—The results described above led us to speculate about the presence of an NES in the coiled-coil region of RFP. In fact, sequence analysis of the coiled-coil region of RFP revealed the putative leucine-rich NES (41, 42). An alignment of the consensus NES (41–45) with the putative leucine-rich NES of RFP indicated a perfect homology (Fig. 3 A). To examine whether RFP contains a functional NES, the three most conserved leucine residues in the putative NES of RFP (Leu-204, Leu-207, and Leu-209 in Fig. 3 A) were mutated to alanines, and the localization of the mutated RFP (RFPmut) as a FLAG-tagged fusion protein was analyzed by immunostaining (Fig. 3 B). RFPmut was located predominantly in the nucleus of NIH3T3 cells and stained with anti-FLAG antibody. In the case of wild-type RFP, transfected cells were also treated with 4 ng/ml LMB for 24 h. FLAG-tagged forms of RFP and DNA staining were visualized through a confocal microscopy as described in Fig. 1B.

NES function can be studied by linking it to nuclear proteins, such as the DNA binding domain of the yeast transcription factor Gal4 (Gal4-DBD), which changes its subcellular localization from the nucleus to the cytoplasm when fused to an NES (47). To study the function of the putative NES in RFP, the full-length RFP and the coiled-coil regions of RFP, comprising either normal or mutated versions of the putative NES, were expressed as Gal4-DBD fusion proteins in NIH3T3 cells, and their subcellular localizations were analyzed using anti-Gal4-DBD antibody (Fig. 4 A). In contrast to the Gal4-DBD control protein, the wild-type RFP fusion protein was localized preferentially to the cytoplasm, and mutations in the putative NES resulted in relocalization of the Gal4-DBD-RFP fusion protein to the nucleus. Similar results were obtained with Gal4-DBD fused to the coiled-coil regions of RFP. The wild-type coiled-coil region of RFP was able to relocate the Gal4-DBD to the cytoplasm. This relocation was again blocked by point mutations in the putative NES. In addition to the cytoplasmic and nuclear localization, fusion proteins of the Gal4-DBD with the coiled-coil region of RFP were found in filament-like structures when the fusion proteins were expressed at very high levels (data not shown). Thus, the NES in the coiled-coil region can function alone.

To confirm the function of the NES in RFP further, we also
fused the RFP protein derivatives to the nuclear form of GFP, which harbors three NLS signals (EGFPnuc). The expression vectors for various EGFPnuc-RFP fusion proteins were transfected into NIH3T3 cells, and their subcellular localizations were analyzed (Fig. 4B). Similar to the results obtained with the Gal4-DBD fusion proteins, wild-type RFP was again able to relocalize EGFPnuc to the cytoplasm. NES mutations led to nuclear localization of the fusion protein to the dot-like structure that resembled those of PML. In contrast, however, fusions of RFPmut with Gal4-DBD were almost uniformly distributed throughout the nucleoplasm (Fig. 4A). This difference could be because, unlike EGFPnuc, Gal4-DBD has DNA binding activity. Because RFP was reported to form nuclear dot-like structure under certain conditions (21), the DNA binding activity of Gal4-DBD may disturb the localization of the fusion proteins to the nuclear dot-like structure. Deletion of the coiled-coil region also resulted in nuclear localization of RFP. However, these fusion proteins were distributed throughout an entire nucleoplasm. Thus, the results obtained with two different nuclear proteins (Gal4-DBD and EGFPnuc) have demonstrated that RFP harbors a functional NES in its coiled-coil region. In mouse, RFP homo-dimerization through the coiled-coil region was reported to be important for RFP localization (18). Human RFP can dimerize in a similar way through its coiled-coil region. Because the NES is in the coiled-coil region of RFP, we investigated whether point mutations in the NES affect the homodimerization using a yeast two-hybrid system.

2 M. Harbers and S. Ishii, unpublished results.
The results showed that wild-type RFP was able to interact with RFPmut, indicating that the point mutations in the NES did not destroy the overall structure of the coiled-coil domain.

The NES Function of RFP Is Masked in HepG2 Cells—In contrast to NIH3T3 cells, RFP is localized predominantly in the nucleus in HepG2 cells. To address whether the NES of RFP is functional in HepG2 cells, the Gal4-DBD-RFP fusion proteins described in the legend of Fig. 4A were expressed in HepG2 cells, and their localizations were analyzed with anti-Gal4-DBD antibody as described in Fig. 4A. Panel B, effect of RFP on the localization of a nuclear form of GFP in HepG2 cells. RFP proteins fused to nuclear GFP (Fig. 4B) were expressed in HepG2 cells, and their localizations were analyzed as described in Fig. 4B.

These results indicate that the activity of NES in full-length RFP is masked by its interaction with other domain(s) in the RFP protein or with other protein(s).
NLS in the C-terminal Portion of RFP—Because RFP protein is localized to the nucleus under certain conditions, RFP protein should also contain an NLS. Therefore, we sought to locate the NLS within RFP. First of all, we determined if a previously described putative NLS (amino acids 156–162) in the N-terminal half of the coiled-coil region of RFP (18) had functional activity (Fig. 6A). We constructed a vector expressing GFP fused to a 527-amino acid region from the *Escherichia coli lacZ* gene product, whose large size precluded its passive diffusion into the nucleus. A fusion protein consisting of GFP-LacZ linked to the N-terminal part of coiled-coil region of RFP (RFP132–169) was not localized predominantly to the nucleus of HepG2 cells. These results indicated that this putative NLS was not active on its own, but other portion(s) or additional portion(s) were required for a functional NLS. To identify the NLS in RFP, we examined the localization of various GFP-RFP fusion proteins in HepG2 cells (Fig. 6B). The GFP fusion protein containing the full-length RFP was localized predominantly in the nucleus of HepG2 cells, indicating that the NES activity of RFP is masked in HepG2 cells and that the NLS of RFP predominantly determines its cellular localization. Deletion of either the Ring finger or the entire RBCC domain, which comprises the RING finger, B box, and coiled-coil region, had no effect on the nuclear localization of GFP-RFP. Furthermore, the GFP fusion containing the RBCC domain of RFP was localized predominantly to the cytoplasm. These results suggested that the NLS was located in the C-terminal portion of RFP. However, no region in the C-terminal portion of RFP displays significant homology with the consensus NLS sequence. Therefore, we were unable to narrow the region of RFP containing the NLS.

Regulation of RFP Localization by PKC Signaling—The NES of RFP is active in NIH3T3 cells but masked in HepG2 cells. This raises the possibility that some specific signals or factors may regulate the NES activity of RFP. We therefore investigated signals that could induce changes in the localization of RFP in NIH3T3 and HepG2 cells. Among the various activators and inhibitors of the different signaling pathways tested, the PKC activator TPA showed the greatest effect (data not shown). Therefore, the effect of PKC-mediated signaling on RFP localization was studied further. Treatment of HepG2 cells with TPA for 1 h induced cytoplasmic localization of RFP in about 55% of cells (Fig. 7A). In these cells, almost complete relocalization of RFP to the cytoplasm was observed, and RFP appeared in dot-like structures similar to those found in NIH3T3 cells. The change in the cellular localization of RFP was only transient because HepG2 cells treated with TPA for 24 h showed nearly exclusive nuclear staining for RFP. To determine whether TPA-induced nuclear export of RFP is dependent on the NES in the coiled-coil domain, HepG2 cells
expressing FLAG-tagged RFP were treated for 1 h with TPA plus LMB. Addition of LMB significantly blocked the release of RFP from the nucleus in HepG2 cells (Fig. 7A). Furthermore, in contrast to wild-type RFP, RFPmut harboring a mutated NES was unable to respond to TPA treatment (Fig. 7A). These results indicate that the NES is required for the TPA-induced relocalization of RFP from the nucleus to the cytoplasm in HepG2 cells.

PKC comprises a growing gene family with up to 12 distinct members. To identify the PKC isoform regulating RFP shuttling, RFP was coexpressed in HepG2 cells together with different PKC isoforms (PKCa, PKCδ, and PKCe) and RFP localization examined (Fig. 7B). Wild-type PKCa had only a minor effect on RFP localization, whereas a constitutively active form of PKCa induced the release of RFP from the nucleus in about 21% of cells with both proteins being localized to the cytoplasm. Cotransfection with either control plasmid or expression vectors for other PKC isoforms had no effect on the nuclear localization of RFP (data not shown).

PKC-mediated nuclear export of RFP was studied further in NIH3T3 and HepG2 cells using different PKC inhibitors. The TPA-induced nuclear export of RFP in HepG2 cells was blocked efficiently by the PKC inhibitors H7 and staurosporine (Fig. 8A). Treatment of NIH3T3 cells with the same inhibitors for 24 h also blocked the nuclear export of RFP and induced nuclear accumulation of RFP, as observed with LMB, without affecting the viability of the cells (Fig. 8B). These results further support our findings that the nuclear export of RFP is positively regulated by PKC activation.

Because RFP showed distinct localizations in NIH3T3 and HepG2 cells, we looked for differences in PKC protein levels and activities between these cell lines (Fig. 9A). Western blot analysis of whole cell extracts from NIH3T3 and HepG2 cells using anti-PKCa and anti-phosphorylated PKCa showed similar amounts of PKCa and phosphorylated PKCa in both cell lines. Similar values were also found for total PKC activity in both cell lines, indicating that the differential pattern of RFP localization was not caused by differences in the amounts or planting.
activities of PKC. Because PKCa has been found in the nucleus of activated NIH3T3 and vascular smooth muscle cells (48–50), the possibility of direct phosphorylation of RFP by PKC was investigated by mutating five putative PKC phosphorylation sites in RFP, identified in a PhosphoBase data base search (51). Neither single mutations (positions T83A, S192A, S290A, T295A, or T453A) nor mutations at all five putative PKC phosphorylation sites together affected RFP localization in NIH3T3 or HepG2 cells, or the TPA-induced nuclear release of RFP in HepG2 cells.2

The similar level of PKC activity in NIH3T3 and HepG2 cells suggested that additional signaling pathways might be also involved in the control of RFP localization. Therefore, the effects of activated Ras and JNK1 on the localization of RFP in HepG2 cells were analyzed further (Fig. 9B). When the activated form of Ha-ras or HA-tagged JNK1 was coexpressed together with FLAG-tagged RFP, a partial release of RFP from the nucleus was observed. Nuclear localization of JNK1 was observed in about a quarter of the transfected HepG2 cells, and cytoplasmic localization of RFP was only observed in cells with nuclear JNK1 (Fig. 9B). In addition, sorbitol treatment of transfected HepG2 cells increased the number of cells with nuclear JNK1 and cytoplasmic RFP.2 These results suggest that JNK1-mediated phosphorylation events in the nucleus result in the nuclear export of RFP. Thus, additional signaling pathways including ras and JNK1 may be involved in the PKC-mediated nuclear export of RFP. Between NIH3T3 and HepG2 cells, the amount or activity of some factors, which act as the transducer of these signaling pathways, may be different.

DISCUSSION

For the control of cell growth and differentiation, sophisticated regulatory mechanisms are necessary for specific and selective responses to various signals. Cells use several distinct mechanisms for signal transduction, including those that alter the cellular localization of individual regulatory factors. Here we studied the cellular localization of RFP in NIH3T3 and HepG2 cells and the regulation of this process in response to external signals. Our study demonstrates that the coiled-coil
domain of RFP containing the leucine-rich NES controls shuttling of RFP between the nucleus and the cytoplasm. The NES was functionally active as RFP containing a mutation in the putative NES accumulated in the nucleus of NIH3T3 cells and the CRM1-specific inhibitor LMB blocked nuclear export of RFP (Fig. 3). This mutated RFP was still able to homodimerize through its coiled-coil region, indicating that the point mutations did not affect the overall structure of the coiled-coil domain but specifically affected the function of the NES. In addition, Gal4-DBD and a nuclear form of GFP (EGFPnuc) were localized to the cytoplasm of NIH3T3 cells when fused to the NES domain of RFP (Fig. 4).

The distinct localization of RFP in NIH3T3 and HepG2 cells argues for tight regulation of the activity of the NES, resulting in only limited exchange between nuclear and cytoplasmic RFP. This regulation might depend on masking of the NES by conformational changes induced by post-translational modification. In most cases, nuclear transport and export are regulated by phosphorylation, as shown for the yeast transcription factor Pho4 (52, 53) and the mammalian factor NF-AT (24). Regulatory domains can also be masked by their binding to other inhibitory factors, as shown for NF-κB (54) and IκB (25, 26), or regulated by homodimerization, which controls the shuttling of mitogen-activated protein kinase (55, 56). As in the case of mitogen-activated protein kinase, RFP dimerizes through its coiled-coil region with potentially important consequences for RFP localization (18). However, it is unlikely that dimerization masks the NES, because mutations in the NES did not affect RFP homodimerization. Another possibility to explain the distinct localization of RFP in NIH3T3 and HepG2 cells is their different diffusion limit. Protein import and export are regulated by a complex machinery, which includes soluble components and nuclear pores (for review, see Ref. 31). Nuclear pores are gated channels composed of at least 50 proteins, many of which have not been characterized. Particles of <9 nm in diameter or globular proteins of less than 50–69 kDa can enter the nucleus by diffusion, whereas larger objects should be actively transported. However, the size limitation for diffusion varies depending on the cell types. Therefore, we cannot completely exclude the possibility that the distinct localization of RFP in NIH3T3 and HepG2 cells partly depends on their different diffusion limit.

RFP localization to the cytoplasm of NIH3T3 cells as well as to the nucleus of HepG2 cells is largely dependent on the

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**Fig. 9.** Effect of various signaling pathways on RFP localization. Panel A, PKC levels in NIH3T3 and HepG2 cells. In the left image, whole cell extracts were prepared from NIH3T3 and HepG2 cells, and 50 μg of total protein from each extract was analyzed by Western blot with anti-PKCα and anti-phosphorylated PKCα-specific antibodies. In the right image, PKC activity in NIH3T3 and HepG2 cells was measured, and the average results of three experiments are indicated by a bar graph. Panel B, effect of various signaling pathways on the subcellular localization of RFP. The FLAG-tagged RFP expression vector was cotransfected with the Ha-ras or JNK1 expression vector or control vectors (pcEXV-1 and pSRα). Immunohistochemistry was performed with an anti-FLAG antibody as described in Fig. 1B, and the percentages of cells with cytoplasmic RFP are shown in the bar graph on the right.
of RFP in NIH3T3 cells because both cell lines had
dence for direct PKC regulation of the differential localization
localization in cell lines other than HepG2. We found no evi-
cells (Fig. 8), suggesting that PKC signaling can control RFP
(63), and PKC is involved in the activation of JNK (64). Al-
though further analysis will be required to identify the mech-
anism through which PKC signaling positively regulates the
nuclear export of RFP, our present results indicate that local-
ization of RFP is regulated by complex processes in a cell type-
specific manner.

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