J. Biochem. **143,** 349–358 (2008)
doi:10.1093/jb/mvm236

Nuclear Localization Signal and Phosphorylation of Serine350 Specify Intracellular Localization of DRAK2

Hiroshi Kuwahara, Michihiko Nishizaki and Hiroshi Kanazawa*

Department of Biological Sciences, Graduate School of Science, Osaka University, Machikaneyama-cho, Toyonaka City, Osaka 560-0043, Japan

Received September 4, 2007; accepted November 13, 2007; published online December 15, 2007

DAP kinase-related apoptosis-inducing kinase 2 (DRAK2) is a serine/threonine kinase of the death-associated protein kinase family. DRAK2 mediates apoptosis induced by extracellular stimuli, including UV irradiation and interleukin-2, and also regulates T-cell receptor sensitivity in developing thymocytes. During these events, the subcellular localization of DRAK2 changes between the nucleus and cytoplasm. We found that DRAK2 has a putative nuclear-localization signal (NLS) sequence. Mutations in this sequence interfered with DRAK2 localization to the nucleus. Furthermore, green fluorescence protein fused to the putative NLS accumulated in the nucleus, indicating that the putative sequence functions as an NLS. We also found that the function of the NLS was regulated by phosphorylation. Phorbol myristate acetate (PMA) induced the accumulation of DRAK2 in the cytoplasm of NIH3T3 cells, whereas in the absence of PMA, DRAK2 was localized to the nucleus. Ectopic expression of PKC-γ induced cytoplasmic localization of DRAK2 and PKC-γ phosphorylated Ser350 flanking the NLS. DRAK2, but not the *Ser350Asp* mutant, accumulated in the nuclei of ACL-15 cells in response to UV-irradiation. These results suggest that phosphorylation of Ser350 plays an essential role in regulating translocation of DRAK2 to the nucleus from the cytoplasm, possibly by affecting the activity of the NLS.

Key words: nuclear localization signal, phosphorylation, protein kinase C (PKC), serine/threonine kinase.

Abbreviations: DRAK2, DAP kinase-related apoptosis-inducing kinase 2; DAPK, death-associated protein kinase; NLS, nuclear localization signal; PMA, phorbol myristate acetate; PKC, protein kinase C; TCR, T-cell receptor.

Death-associated protein (DAP) kinase-related apoptosis-inducing protein kinase 2 (DRAK2), a serine/threonine kinase, was originally identified as a pro-apoptotic protein kinase composed of a kinase domain and a DRAK2-specific domain (*1*, *2*). The kinase domain shares a sequence with other DAP kinase family members. Although the physiological functions of DAP kinases have been extensively examined (*3*), DRAK2 has not been well studied. Since DRAK2 is expressed in thymus, testis, spleen and brain, it may play a role in these tissues (*2*). T cells derived from a DRAK2-knockout mouse are highly sensitive to T-cell stimuli, but do not show any defect in T-cell apoptosis (*4*). Further studies using transgenic mice have suggested that DRAK2 enhances T-cell apoptosis by stimulation of interleukin-2 synthesis (*5*). Thus, although DRAK2 appears to have a physiological role in the thymus, various facets remain to be elucidated, including the significance of its expression in other organs.

The function of DRAK2 remains unclear at both the organismic and the cellular level. Cultured cell lines are useful tools for addressing the cellular function of DRAK2. However, we have shown that the intracellular localization of DRAK2 varies depending on the cell line (*6*). DRAK2 is primarily localized to the nucleus in NRK cells, while DRAK2 is primarily localized to the cytoplasm in the rat colon carcinoma cell line ACL-15. Furthermore, DRAK2 localization is affected by extracellular stimuli. In Jurkat cells, treatment with phorbol myristate acetate (PMA) and phytohaemagglutinin (PHA) shifts DRAK2 localization from nucleus to the cytoplasm (*7*). In ACL-15 cells, UV irradiation (an apoptosis inducer) causes translocation of DRAK2 from the cytoplasm to the nucleus and subsequently induces apoptosis (*6*). We also showed that DRAK2 in the nucleus plays a role in cell death, but that DRAK2 in the cytoplasm may not. Although the intracellular localization of DRAK2 is important for determining cell fate, the molecular mechanisms that determine its subcellular localization are poorly understood.

A nuclear-localization signal (NLS) is one of factors regulating protein localization to the nucleus. The NLS is a short basic sequence that is required for the active import of proteins into the nucleus (*8*). DRAK2 has several clusters of the basic amino acids, lysine and arginine, that are NLS candidates. In addition to these putative NLS sequences, residues whose phosphorylation might regulate the putative NLS have been identified. It has been reported that the subcellular localization of SV40 large tumour antigen is controlled by the phosphorylation state

*To whom correspondence should be addressed. Tel: +81 6 6850 5812, Fax: +81 6 6850 5812,
E-mail: kanazawa@bio.sci.osaka-u.ac.jp

Table 1. **Oligonucleotides used in Fig. 1.**

Deletion		Sequence (5′-3′)
aa 1–356	(FW)	ATTCTCAAGCCTCAGACAGT
	(RV)	ATTTCGATTCGATGAC**TAG**GGATCCAGC (1)
aa 1–345	(FW)	ATTCTCAAGCCTCAGACAGT
	(RV)	GAACATCCCTGAGGAT**TAG**GATCCACTT (1)
aa 34–371	(FW)	CGGGATCCCCTTACCCCGAAAGAACTTGGGAGA (1)
	(RV)	GGGAGACCTGATACTCTCAA
aa 34–293	(FW)	CGGGATCCCCTTACCCCGAAAGAACTTGGGAGA (1)
	(RV)	CTTTCCCACTCATGGCTG**TAG**TCGACGGAC (2)

*Bam*H I (1) and *Sal* I (2) restriction sites are underlined. A second BamH 1 site was located in the expression plasmids but not in the primers. Bold letters indicate stop codons. (FW) and (RV) indicate forward and reverse primers, respectively.

of Ser112 and Ser120 (*9*, *10*). Phosphorylation often increases or decreases the interaction between the NLS and importins, which act to carry proteins through nuclear pores (*11*).

In this study, we find that DRAK2 has a canonical NLS in its C-terminal region (aa 294–371). We also found that Ser350 is a phosphorylation site for protein kinase C (PKC)-γ, and that its phosphorylation affects the nuclear localization of DRAK2. These results support a scenario in which DRAK2 has a functional NLS whose activity is regulated by PKC phosphorylation of Ser350.

MATERIALS AND METHODS

Cell Culture and Transfection—COS-7 cells and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% CO_2. ACL-15 cells were maintained in RPMI-1640 medium supplemented with 10% FBS at 37°C in 5% CO_2.

Cells were transfected with expression vectors encoding DRAK2 variants, using the cationic lipid reagent TransIT LT-1 (Mirus) for COS-7 and NIH3T3, FuGene6 (Roche Applied Science) for ACL-15 cells, according to the Manufacturer's instructions.

Immunofluorescence Microscopy—Twenty-four to 48 h after transfection, cells were fixed for 25 min at room temperature with 2% paraformaldehyde in phosphate-buffered saline (PBS). For immunostaining with monoclonal anti-Myc tag antibodies (Santa Cruz), fixed cells were permeabilized by incubation in PBS containing 0.4% saponin for 15 min and blocked with 1.0% bovine serum albumin (PBS/BSA). The cells were incubated with monoclonal anti-Myc tag antibodies in PBS/BSA for 1 h at room temperature and then washed with PBS. The cells were then incubated with Alexa-546-conjugated anti-mouse IgG (Molecular Probes) in PBS/BSA for 1 h and stained with Hoechst 33342 (0.1 ng/ml in PBS) at 37°C. Coverslips were mounted using 50% glycerol/PBS. Immunofluorescence was detected under a fluorescence microscope (Olympus BX51) equipped with an ORCA-ER digital camera (Hamamatsu Photonics).

Immunoblotting—Cells transfected with Myc-tagged DRAK2 or its derivatives were collected in lysis buffer [20 mM Tris–HCl, pH 7.4, 0.25 M NaCl and protease inhibitor cocktail (Roche Applied Science)]. Following centrifugation at 1,000*g* for 3 min, the precipitated fraction was used as the total protein lysate. Total cellular proteins were subjected to SDS–PAGE and the separated proteins were transferred to nylon membranes as described previously (*12*). Each membrane filter was incubated with anti-Myc antibody (Clontech) for detection of the transfected proteins. Immunoreactive materials were visualized by the ECL chemiluminesence method according to the manufacturer's instructions (GE Healthcare).

Construction of Expression Plasmids—A rat Myc-DRAK2 expression vector and Myc-DRAK2 (aa 1–293) expression vector were constructed as described previously (*2*). Constructs encoding truncations of Myc-DRAK2 (aa 1–356, aa 1–345 and aa 34–371) were created by amplification of the Myc-tagged DRAK2 open reading frame (ORF) with appropriate primers as described in Table 1. All amplified DNAs were inserted into the *Bam*H I restriction site of the Myc-DRAK2 expression vector. Myc-DRAK2 (aa 34–293) was amplified from Myc-DRAK2 (aa 1–293) and inserted into the *Bam*H I/*Sal* I sites of the Myc-DRAK2 (*1–293*) expression vector. Expression plasmids encoding DRAK2 with alanine or asparatic-acid mutations (putative NLS, S347D, S350D, S357D, S360D and S350A) were prepared by PCR-based mutagenesis using primers with the corresponding mutations as described previously (*13*) (Table 2). In short, the Myc-DRAK2 expression plasmid was amplified using primer pairs that included a point mutation. Expression plasmids for green fluorescence protein (GFP)-fusion proteins with the NLS or its derivative were constructed as follows. Oligonucleotide corresponding to the sequence of the NLS (EDDSLLSKRFRFDDSLPSPHE) was synthesized with *Eco*R I sites at their 5′ and 3′ ends (Invitrogen). This sequence was inserted in-frame into the expression vector pEGFP-N (BD Clontech). The primary sequences of all constructs were confirmed by sequencing with an automatic DNA sequencer (Applied Biosystems).

Table 2. **Oligonucleotides used in Figs 2 and 3.**

Mutation	Sequence (5′-3′)	Codon change
KKRRR→KNQQR	ATTCCTGAAGAATCAGCAGAGAGGGCAGG	AAGAGGAGA→AATCAGCAG
KRFR→AAFA	CCCTGAGGATGACAGCTTACTTTCTGCAGCA	AAAAGA→GCAGCA
	TTTGCATTCGATGACTCCTTGCCCAGCCCCCAC	CGA→GCA
S347D	GGAGAACATCCCTGAGGATGACGACTTACTTTCTAAAAGA	AGC→GAC
S350D	CCCTGAGGATGACAGCTTACTTGACAAAAGATTTCGATTC	TCT→GAC
S357D	CGATTCGATGACGACTTGCCCAGC	TCC→GAC
S360D	GACTCCTTGCCCGATCCCCACGAA	AGC→GAT
S350A	CCCTGAGGATGACAGCTTACTTGCTAAAAGATTTCGATTC	TCT→GCT

The mutated sequences of the target codon are underlined.

The expression plasmid for PKC-γ was constructed as described previously (*14*).

Phosphorylation of DRAK2 and the Synthetic Peptides by PKC—Phosphorylation of the synthetic peptide corresponding to aa 345–355 of DRAK2, CDDSLLSKRFRF (Invitrogen), was performed by incubating 0.31 μg of PKC-γ (Invitrogen) and 80 nmol (4 mM) of the peptide in kinase buffer [20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 100 μM phosphatidyl serine, 10 μM PMA and 0.1 mM [γ-^{32}P]ATP (111 GBq/mmol; Amersham Pharmacia)] in a total volume of 20 μl at 30°C for 30 min. The control peptide, which lacked the phosphorylation site (CDDSLLAKRFRF), was also generated. The reaction mixture was spotted onto a 1 cm diametre Whatman P81 cellulose-phosphate filter circle. After washing with 75 mM phosphoric acid, the P81 filter was dried and subjected to autoradiography. The radioactive phosphopeptides were detected with an auto-imaging analyser (BAS1800, Fuji Film) and quantified using ImageGauge software (version 3.41, Fuji Film).

RESULTS

DRAK2 has a Functional NLS Sequence in Its C-terminal Region—Previous studies showed that DRAK2 localized to the nuclei of NIH3T3, NRK and COS7 cells, and that the C-terminal region (aa 294–371) was required for nuclear localization (*6*). To more precisely identify the regions of DRAK2 that are responsible for its nuclear localization, we analysed the intracellular localizations of full-length and various truncated forms of DRAK2 in NIH3T3 cells (Fig. 1A). NIH3T3 and COS7 cells show a similar pattern of DRAK2 nuclear localization, suggesting that mechanisms of DRAK2 nuclear localization in these two cell lines may be the same. Plasmids expressing deletion derivatives of DRAK2 were transfected into NIH3T3 cells and their localizations were analysed 36 h after transfection. We confirmed that wild-type DRAK2 was located primarily in the nucleus of NIH3T3 cells (Fig. 1B, wild-type). Among the DRAK2 derivatives shown in Fig. 1A, regions 1–356 and 34–371 localized to the nucleus similarly to the wild type, whereas regions 1–293 and 1–345 did not localize to the nucleus (Fig. 1B). These results suggested that the region encompassing amino acids 346–356 of DRAK2 were involved in nuclear localization. The DRAK2-derivative proteins migrated at their expected molecular sizes on SDS–PAGE (Fig. 1C). The region involved in nuclear localization contained one stretch of basic amino acids (KRFR, aa 351–354), similar to a canonical NLS, suggesting that this region might be responsible for the nuclear localization.

In order to determine whether the basic amino acids in the putative NLS (aa 351–354) were responsible for the nuclear import of DRAK2, we examined the localization of Myc-DRAK2 with the lysine and arginine residues replaced by alanine (Table 2, KRFR→AAFA). In contrast to wild-type DRAK2, which was located predominantly in the nucleus, this mutated DRAK2 did not accumulate in the nucleus, but was scattered throughout the NIH3T3 cells (Fig. 2A). The mutant protein was similar in size to wild-type DRAK2 (Fig. 2B). These results demonstrated the requirement for these basic amino acids (KRFR, aa 351–354) in a functional NLS for DRAK2 nuclear localization.

To further demonstrate the putative sequence as a NLS, we tested whether this sequence could cause the nuclear localization of GFP. Whereas GFP alone localized to both the nucleus and the cytoplasm of COS-7 cells (Fig. 2C), the GFP–DRAK2 NLS fusion (GFP–NLS) localized to the nucleus, indicating that this region (aa 344–363) contained a functional signal sequence that was sufficient for nuclear import. Based on these two different lines of experimental evidence, we concluded that the putative NLS in the C-terminal region is responsible for the nuclear localization of DRAK2.

It was reported previously that a sequence located near the kinase catalytic domain of DRAK2 functioned as an NLS in Jurkat cells (aa 65–69) (*7*). Our present results demonstrated that a functional NLS sequence is located in the C-terminal region (aa 294–371) when it was expressed in NIH3T3 cells. To examine whether the region (aa 65–69) also functions as an NLS sequence in NIH3T3 cells, we expressed a Myc-tagged DRAK2 derivative, in which KKRRR (aa 65–69) was replaced with KNQQR. This mutant derivative localized primarily to the nuclei, suggesting that this region (aa 65–69) did not necessarily function as a NLS sequence to cell type (Fig. 2D and E). Therefore, we concluded that the KRFR sequence in the C-terminal region is primarily required for nuclear localization of DRAK2.

NLS Function of DRAK2 is Regulated by Phosphorylation of Ser350 by PKC—Since DRAK2 had been shown to localize to the nucleus or cytoplasm under different physiological conditions, it was expected that the localization might be regulated by alterations in NLS function. The phosphorylation-prediction program Net Phosk 1.0 gave the highest PKC phosphorylation score

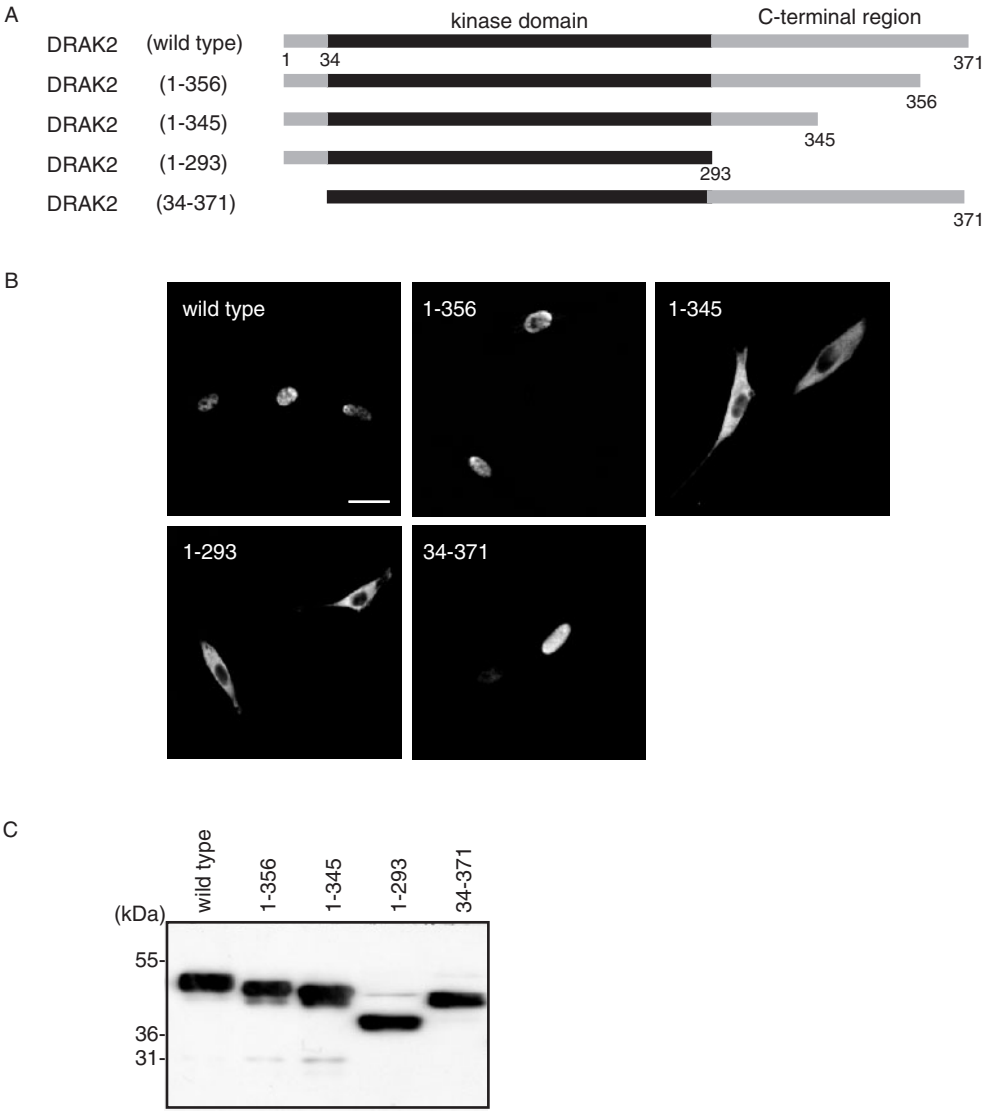

Fig. 1. **Subcellular distribution of various deletion mutants of DRAK2.** (A) Schematic representation of full-length DRAK2 and a series of DRAK2-deletion constructs. The constructs are deletions of the DRAK2 N-terminal and/or C-terminal regions, other than the kinase catalytic domain. (B) Expression plasmids encoding wild-type DRAK2 or the deletion mutants were transfected into NIH3T3 cells. Thirty-six hours after transfection, the cells were fixed and stained with anti Myc-tag antibody. DRAK2 was visualized by utilizing Alexa fluor-546-conjugated anti-mouse IgG. Representative images of a large number of cells from three independent experiments are presented. The scale bar corresponds to 20 μm. (C) Cells transfected with the DRAK2-expressing plasmids shown in (A) were subjected to SDS–PAGE. The proteins were transferred to a GVHP filter and probed with anti-Myc monoclonal antibody.

to Ser350, which is adjacent to the NLS (KRFR) (*15*). Therefore, we examined whether DRAK2 was phosphorylated by PKCs. Among PKC isoforms, we found that PKC-γ phosphorylated DRAK2 with the highest efficiency (data not shown). We next analysed the phosphorylation of Ser350 by PKC-γ. A synthesized peptide corresponding to amino acids 345–355 of DRAK2 and a control peptide, in which Ser350 was replaced with alanine, were subjected to phosphorylation with [γ-^{32}P]-ATP. The wild-type peptide was radiolabelled, whereas the control peptide was not significantly phosphorylated in comparison with the mock control, indicating that Ser350 is phosphorylated by PKC-γ (Fig. 3).

We next tested whether this phosphorylation of Ser350 near the NLS sequence influenced nuclear import of DRAK2. Myc-DRAK2 derivatives containing replacements of serine residues 347, 350, 357 or 360 with aspartic acid were generated (Fig. 4A). Aspartic acid is known to mimic the negative charge of the phosphorylated side chain (*11*). Replacement of serine residues 347, 357 or 360 with aspartic acid did not affect localization. However, NLS derivatives with a substitution at Ser350 attenuated the nuclear localization of Myc-DRAK2 (Fig. 4B),

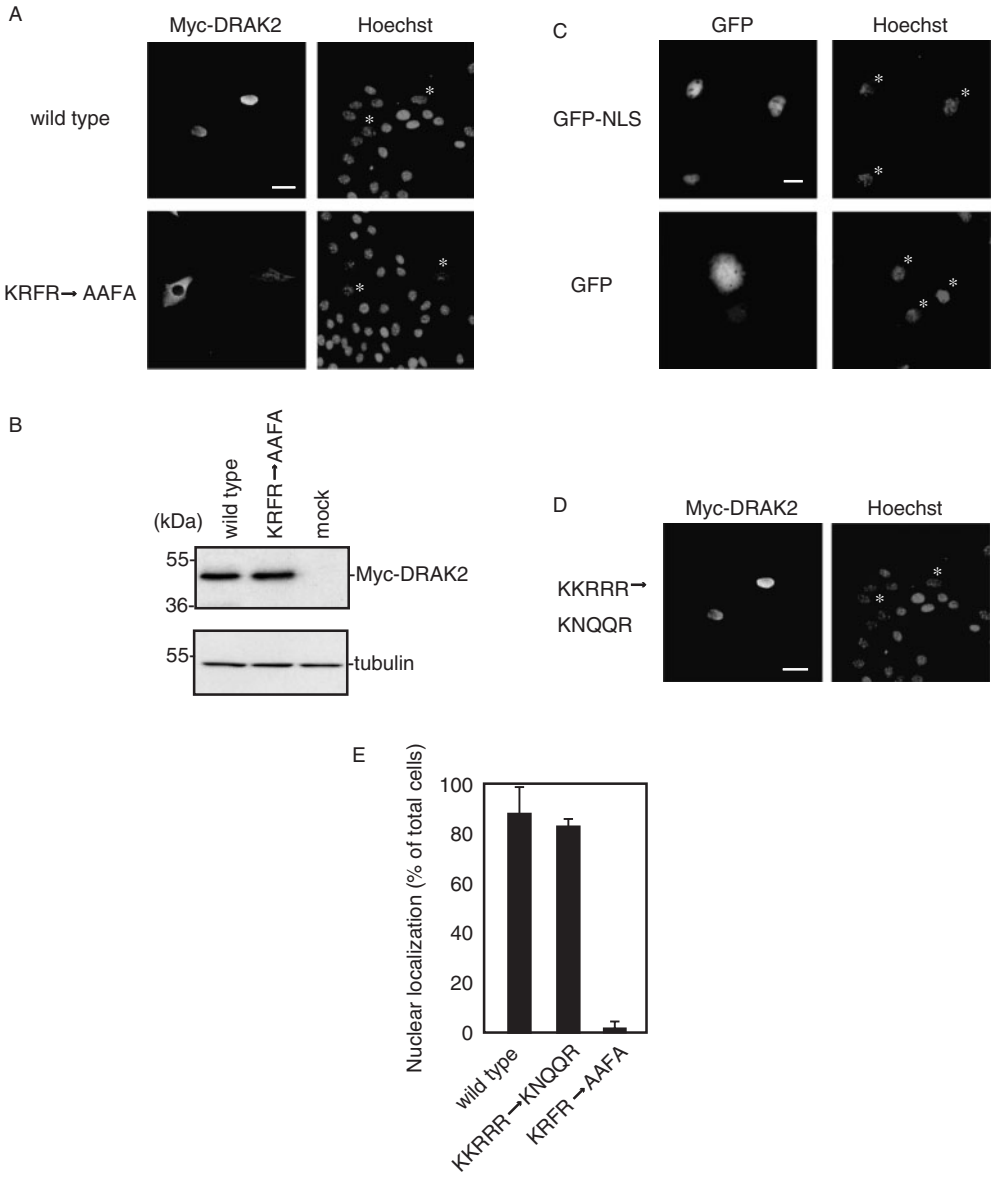

Fig. 2. **DRAK2 has a functional NLS sequence in its C-terminal region (aa 294–371).** (A) DNA constructs encoding Myc-tagged wild-type DRAK2 or DRAK2 with an NLS mutation (KRFR→AAFA) were transfected into NIH3T3 cells. The subcellular distributions of the protein products were determined as described in the MATERIALS AND METHODS section. Myc-DRAK2 was visualized by Alexa fluor-546-conjugated anti-mouse IgG (left) and nuclei were stained with Hoechst 33342 (right). Myc-DRAK2-transfected cells are indicated by white asterisks. The scale bar corresponds to 20 μm. (B) DRAK2 transfected cells (wild-type, KRFR→AAFA or mock) were harvested and subjected to SDS–PAGE, followed by immunoblotting with anti Myc-tag antibody (top panel) and anti tublin antibody (bottom panel). (C) A peptide corresponding to amino acids 344–363 including the putative NLS sequence of DRAK2 was fused to the C-terminus of GFP. Expression vectors containing this construct or GFP alone were transfected into COS-7 cells and, 48 h after transfection, the subcellular distribution of the fusion proteins were observed by fluorescence microscopy (left). Nuclei were also stained with Hoechst 33342 (right). White asterisks indicate transfected cells. The scale bar corresponds to 20 μm. (D) NIH3T3 cells were transfected with a Myc-tagged DRAK2 derivative in which KKRRR (aa 65–69) was replaced with KNQQR. The transfected cells were observed by indirect immunofluorescence of Myc-tag DRAK2 (left) and Hoechst 33342 staining (right). White asterisks indicate transfected cells. The scale bar corresponds to 20 μm. (E) NIH3T3 cells were transfected with wild-type DRAK2 and the *KKRRR→KNQQR* and *KRFR→AAFA* mutant constructs. After 36 h, the numbers of cells expressing Myc-tagged DRAK2 primarily in the nuclei were counted and presented as percentages of the total number of cells; more than 100 cells were counted. Values represent the means obtained from three independent experiments.

suggesting that phosphorylation of Ser350 is required for cytoplasmic localization of DRAK2.

We further examined the correlation between Ser350 phosphorylation by PKC and subcellular localization by observing the effects of PMA on DRAK2 localization. Without PMA treatment, DRAK2 was observed primarily in the nucleus, whereas with PMA DRAK2 was found in cytoplasm (Fig. 5). Myc-DRAK2 with an alanine

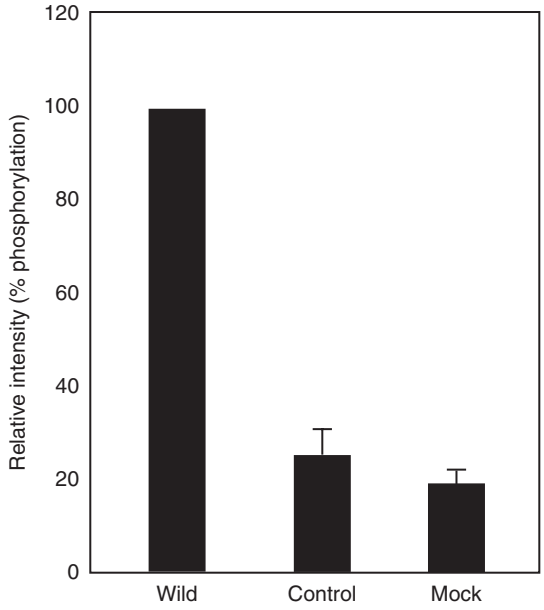

Fig. 3. **PKC phosphorylation of Ser350 of DRAK2.** A peptide corresponding to amino acids 345–355 of DRAK2 and a control peptide, in which Ser350 was replaced with Ala, were generated. The peptides or control buffer alone (mock) were incubated with PKC-γ and [γ-^{32}P]ATP for 30 min. Phosphorylated peptides were detected as described in the MATERIALS AND METHODS section. The relative intensity of phosphorylation is shown as a percentage of the wild-type peptide phosphorylation (mean ± SD; $n = 3$).

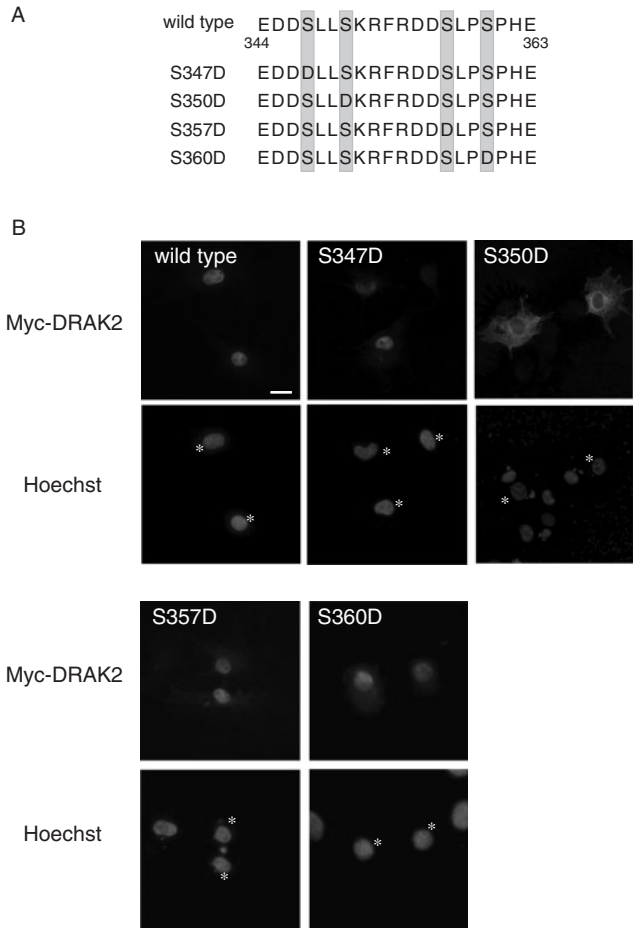

Fig. 4. **NLS activity by modulation of amino-acid charge.** (A) The four serine residues near the NLS sequence of DRAK2, between aa 344 and 363, were replaced with aspartic acids (grey boxes). (B) DRAK2 expression plasmids containing the substitutions in (A) were introduced into COS-7 cells. Thirty-six hours after transfection, the subcellular distributions of the Myc-DRAK2 derivatives were visualized by indirect immunofluorescence. Nuclei were also stained with Hoechst 33342. Myc-DRAK2-transfected cells are indicated by white asterisks. The scale bar corresponds to 20 μm.

substitution at Ser350 did not localize to the cytoplasm following PMA treatment, suggesting that Ser350 phosphorylation was induced by the PKC pathway. Since it has been known that PMA activates many kinases (*16–18*), kinases other than PKCs may influence DRAK2 localization. To examine the effect of PKC on DRAK2 localization more preciously, we co-expressed PKC-γ with DRAK2 (wild-type or S350A), and observed intracellular localization of both of these proteins. In agreement with the data shown in Fig. 1, DRAK2 was detected mainly in nuclei; however, when PKC-γ was overexpressed, DRAK2 was detected more in the cytoplasm, suggesting that PKC-γ influences DRAK2 localization (Fig. 6). On the other hand, the localization of DRAK2 with an alanine substitution at Ser350 was not influenced by PKC expression, confirming that PKC is involved in Ser350 phosphorylation and regulation of the NLS activity of DRAK2. These results suggest that activated PKC can phosphorylate DRAK2, causing a change in its subcellular localization. In Fig. 5C, we observed that about 30% of the cells showed nuclear localization of DRAK2 after 90 min of PMA treatment. However, since exposure of the cells to PMA for > 90 min caused significant morphological changes of the cells, it is difficult to draw any firm physiological implications from this result.

Nuclear Translocation of DRAK2 upon UV Irradiation is Regulated by Ser350—We previously showed that nuclear translocation of DRAK2 occurred in ACL-15 cells after UV irradiation and subsequently induced apoptosis (*6*). However, the precise mechanisms of this nuclear translocation were not examined. We hypothesized that the NLS and phosphorylation of Ser350 might regulate this UV-sensitive process. ACL-15 cells transfected with the wild type, the NLS derivative KRFR→AAFA, or the phosphomimic form (S350D) of DRAK2 were UV-irradiated. Eight hours after irradiation, the cells were fixed and the intracellular distribution of Myc-DRAK2 was observed by immunostaining (Fig. 7). The level of wild-type Myc-DRAK2 was increased in the nucleus after UV irradiation, although the mutant DRAK2s (*KRFR→AAFA* and *S350D*) remained in the cytoplasm. These results suggest that the NLS and non-phosphorylated Ser350 are required for nuclear translocation of DRAK2 following UV irradiation.

DISCUSSION

We and another group reported previously that DRAK2 is localized to the cytoplasm or the nucleus depending on the

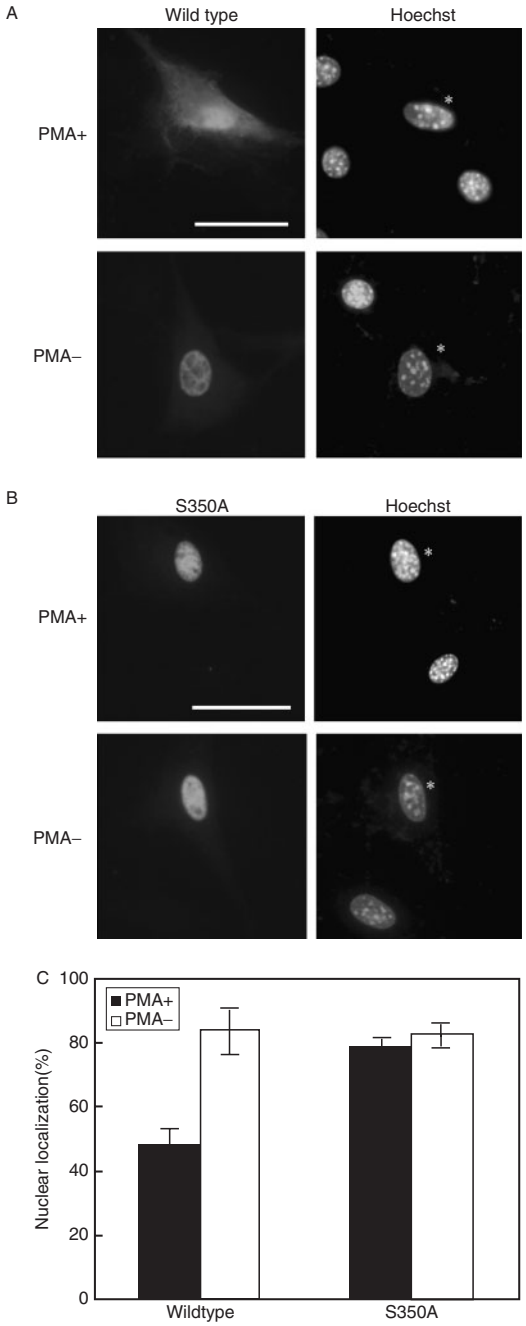

Fig. 5. **The effects of PMA treatment on the subcellular localization of DRAK2.** (A) NIH3T3 cells were transfected with Myc-DRAK2. After 36 h, cells were left untreated or treated with 50 nM PMA for an additional 60 min. The cells were fixed and stained with anti-Myc antibody and visualized with Alexa fluor-546 anti-mouse IgG (left). Nuclei were stained with Hoechst 33342 (right). White asterisks indicate Myc-DRAK2-transfected cells. The scale bar corresponds to 20 μm. (B) Ser 350 of DRAK2 was replaced by alanine. Cells were transfected with this DRAK2 derivative, and 60 min after treatment with or without 50 nM PMA, the cells were visualized as described in (A). White bar corresponds to 20 μm. (C) Numbers of NIH3T3 cells showing predominantly nuclear localization of wild-type and S350A mutant *DRAK2* were counted at 90 min after PMA treatment. Closed bars, PMA-treated; open bars, untreated. Data are the means ± SD of three independent experiments; >100 cells were counted in each experiment.

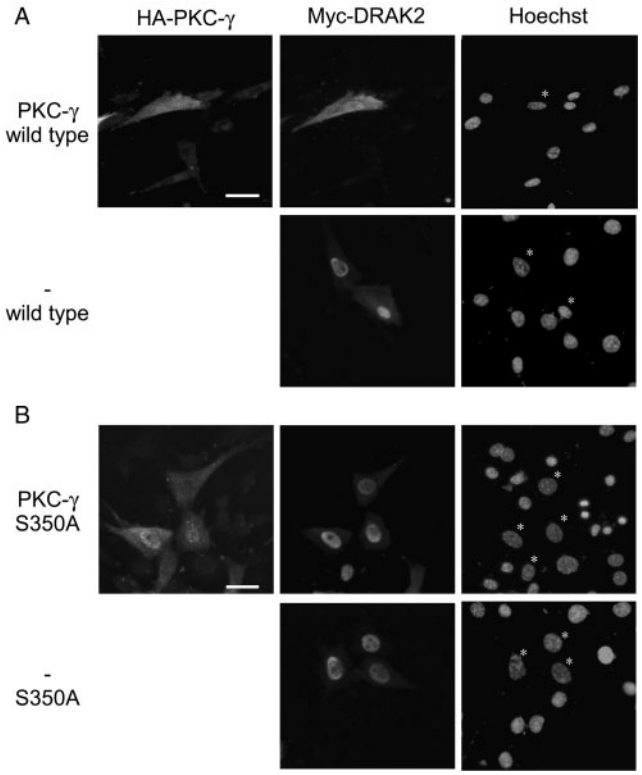

Fig. 6. **Effect of PKC-γ expression on DRAK2 nuclear translocation.** (A) Wild-type DRAK2 was co-expressed with or without PKC-γ in NIH3T3 cells. After chemical fixation, cells were stained by a HA-tag specific antibody and by a Myc-tag specific antibody. The immunocomplexes were visualized with alexa 488-conjugated anti-rabit IgG (left) and alexa 546-conjugated anti-mouse IgG (middle), respectively. Nuclei were stained by Hoechst 33342 (right). White asterisks indicate myc-DRAK2-transfected cells. Scale bar corresponds to 20 μm. (B) The mutant *DRAK2* with an alanine substitution at Ser350 was expressed in NIH3T3 cells with or without PKC-γ. PKC-γ (left) and DRAK2 derivative (middle) were visualized by indirect immunofluorescence as in (A). Nuclei were stained by Hoechst33342 (right).

cell line or physiological conditions, and that the carboxy-terminal region (aa 294–371) is required for nuclear localization (*6*, *7*). Although the localization of DRAK2 is important for its function, the precise mechanisms were previously unknown. In the present study, we showed that DRAK2 has a new functional NLS (KRFR) in its C-terminal region by examining the localizations of GFP–KRFR fusions containing alanine substitutions within the KRFR sequence. We further showed that DRAK2 localization to the nucleus is controlled by the phosphorylation state of Ser350. This was shown by performing *in vitro* phosphorylation assays with PKC and mutagenic analyses of Ser350, as well as by examining the effect of PMA and PKC expression on DRAK2 localization. These findings imply that the intracellular localization of DRAK2 is regulated by its NLS sequence and Ser350 phosphorylation.

Friedrich *et al*. (*7*) showed previously that the sequence KKRRR (aa 65–69) in the DRAK2 kinase domain functions as an NLS for DRAK2 in Jurkat cells.

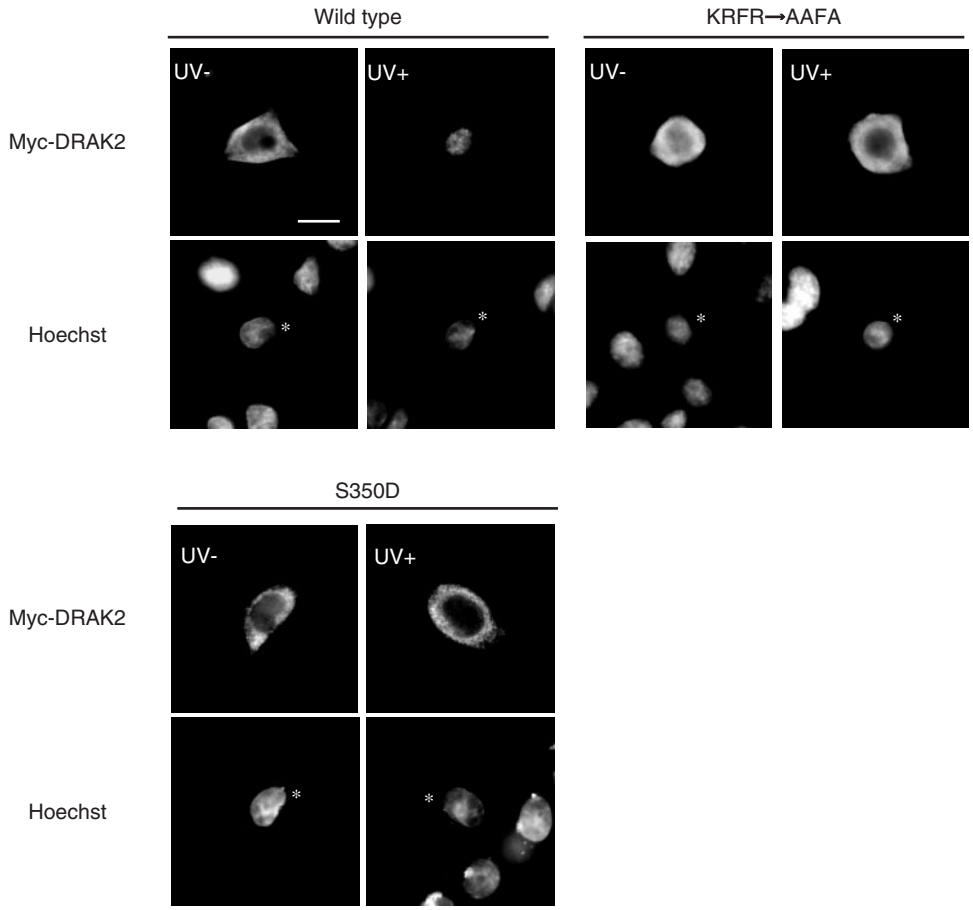

Fig. 7. **UV-induced translocation of DRAK2 requires the NLS and unphosphorylated Ser350.** ACL-15 cells were transfected with pEF-BOS-EX vectors containing Myc-tagged *DRAK2* genes (wild-type, KRFR→AAFA or S350D). After 36 h, the cells were UV-irradiated and incubated for another 8 h. The subcellular distributions of Myc-DRAK2 were detected using Alexa fluor-546-conjugated anti-mouse IgG (upper). The nuclei were stained with Hoechst 33342 (lower). White asterisks indicate transfected cells. The scale bar corresponds to 20 μm.

Our results indicate that the sequence KRFR in the C-terminal region is also required for nuclear localization (Fig. 2). Since canonical NLS sequences consist of short stretches of basic amino acids, such as PKKKRKV in the NLS of SV40, both sequences, KKRRR and KRFR, could function as NLS in DRAK2 (*19*). However, we could not find evidence to indicate that KKRRR is a functional NLS in NIH3T3 cells. This discrepancy might reflect differences in the molecular mechanisms regulating the nuclear localization of DRAK2 between NIH3T3 and Jurkat cells. In this article, we showed that C-terminal NLS activity is regulated by Ser350 phosphorylation. KKRRR in the kinase domain might also be regulated by unknown mechanisms. Future studies will be directed to uncovering any functional correlations between these two potential NLS, including the possibility that these NLS are regulated by the phosphorylation of nearby sequences.

Although each member of the DAP kinase family shares a similar kinase domain, other domains, as well as subcellular localization, differ considerably among family members. Whereas ZIP kinase is known to be a nuclear protein, DAPK is localized in the cytoplasm (*3*). ZIP kinase has a functional NLS sequence outside of the kinase catalytic domain, and DAPK has a cytoskeletal-binding domain in the C-terminal region (*20*, *21*). These observations suggest that the C-terminal region may play an important role in subcellular localization among DAP kinase family members. This hypothesis is consistent with the fact that the DRAK2 NLS is in the C-terminal region.

It is well known that phosphorylation can regulate the nuclear transport process by directly modulating NLS sequence function (*22*), as has been shown for the NLS of SV-40 that is regulated by phosphorylation with CK2, dsDNA-PK and PK-A (*10*, *22*, *23*). FMIP, the M-CSF receptor-interacting protein, is phosphorylated by PKC at Ser5 and Ser6. These serines are adjacent to the NLS (KRKK) and their phosphorylation prevents translocation of FMIP to the nucleus (*24*). *In vitro* phosphorylation and the change in localization due to PMA treatment suggests that, as is the case for FMIP, the nuclear localization of DRAK2 is negatively regulated by PKC phosphorylation.

We showed previously that kinase activity is required for nuclear localization of DRAK2 since kinase inactive DRAK2 was scattered throughout whole cells (*6*). This result suggests that not only PKC but also DRAK2 itself

might be involved in regulating its subcellular localization. Recently, Ser348 of human DRAK2, corresponding to Ser347 of rat DRAK2, has been reported to be subject to auto-phosphorylation (*25*). From these results, it appears likely that C-terminal NLS activity may be regulated by the phosphorylation of not only Ser350 but also of Ser347. The phosphorylation of Ser350 by PKC might be difficult if Ser347 has already been auto-phosphorylated as an existing phosphate-amino acid could potentially inhibit the phosphorylation of another closely located amino acid through unfavourable steric and electrical interactions. Conversely, phosphorylated Ser350 could prevent auto-phosphorylation of Ser347, resulting in the sequestration of DRAK2 in the cytoplasm. Currently, we do not know precisely which conditions favour the phosphorylation of Ser347 or Ser350. The intracellular calcium concentration may be an important element governing this choice, because DRAK2 kinase activity is controlled by a multifunctional calcium binding protein, CHP (calcineurin B homologous protein), and PKC is activated by calcium (*26–28*). When the calcium concentration is high enough ($Ca^{2+} > 10^{-6}$ M), autophosphorylation of serine 347 could be suppressed by activated CHP, resulting in accelerated phosphorylation of Ser350. This putative series of events could lead to the sequestration of DRAK2 in the cytoplasm. Further study of these events will be required to determine whether the above conjecture is correct or not.

We have previously shown that, upon UV-induced apoptosis, DRAK2 is translocated from the cytoplasm to the nucleus in cells such as ACL-15 (*6*). The experiments using a phosphomimetic substitution of Ser350 suggest that DRAK2 nuclear-cytoplasmic shuttling is prevented by phosphorylation (Fig. 7). It is known that UV-induced apoptosis also activates various kinases including JNK, p38MAPK and PKC-δ (29–31). Therefore, DRAK2 might be phosphorylated by certain of these kinases, but not by PKC-δ, which did not phosphorylate DRAK2 in an *in vitro* assay (data not shown). Since DRAK2 translocation to the nucleus coincided with the induction of apoptosis, it seems likely that Ser350 is phosphorylated. If this was the case, phosphorylated Ser350 would have to be dephosphorylated eventually. In this context, it is worth remarking that UV irradiation causes de-phosphorylation of NFAT by calcineurin, resulting in NFAT translocation to the nucleus (*32*). Another possibility is that the phosphorylation of DRAK2 by UV-induced kinases might result in DRAK2 degradation by the ubiquitin–proteasome pathway, resulting in only newly synthesized and unphosphorylated DRAK2 being translocated to the nucleus. This latter idea is partly supported by our preliminary results showing that DRAK2 is partially ubiquitinated (data not shown). Further studies will be required to elucidate how PKC affects DRAK2 during UV-induced apoptosis. The present findings provide new clues for our understanding of DRAK2 function *in vivo*, including in the immune system and in the development of cancer.

We thank Dr Shun'ichi Kuroda (Osaka Univeristy) for kindly providing us expression plasmid of PKCs. This study was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, Technology and Culture of Japan.

REFERENCES

1. Sanjo, H., Kawai, T., and Akira, S. (1998) DRAKs, novel serine/threonine kinases related to death-associated protein kinase that trigger apoptosis. *J. Biol. Chem.* **273**, 29066–29071
2. Matsumoto, M., Miyake, Y., Nagita, M., Inoue, H., Shitakubo, D., Takemoto, K., Ohtsuka, C., Murakami, H., Nakamura, N., and Kanazawa, H. (2001) A serine/threonine kinase which causes apoptosis-like cell death interacts with a calcineurin B-like protein capable of binding Na^+/H^+ exchanger. *J. Biochem.* **130**, 217–225
3. Shohat, G., Shani, G., Eisenstein, M., and Kimchi, A. (2002) The DAP-kinase family of proteins: study of a novel group of calcium-regulated death-promoting kinases. *Biochim. Biophys. Acta* **1600**, 45–50
4. McGargill, M.A., Wen, B.G., Walsh, C.M., and Hedrick, S.M. (2004) A deficiency in Drak2 results in a T cell hypersensitivity and an unexpected resistance to autoimmunity. *Immunity* **21**, 781–791
5. Mao, J., Qiao, X., Luo, H., and Wu, J. (2006) Transgenic drak2 overexpression in mice leads to increased T cell apoptosis and compromised memory T cell development. *J. Biol. Chem.* **281**, 12587–12595
6. Kuwahara, H., Nakamura, N., and Kanazawa, H. (2006) Nuclear localization of the serine/threonine kinase DRAK2 is involved in UV-induced apoptosis. *Biol. Pharm. Bull.* **29**, 225–233
7. Friedrich, M.L., Wen, B.G., Bain, G., Kee, B.L, Katayama, C., Murre, C., Hedrick, S.M., and Walsh, C.M. (2005) DRAK2, a lymphoid-enriched DAP kinase, regulates the TCR activation threshold during thymocyte selection. *Int. Immunol.* **17**, 1379–1390
8. Smith, A.E., Kalderon, D., Roberts, B.L., Colledge, W.H., Edge, M., Gillett, P., Markham, A., Paucha, E., and Richardson, W.D. (1985) The nuclear location signal. *Proc. R. Soc. Lond. B Biol. Sci.* **226**, 43–58
9. Xiao, C.Y., Hubner, S., and Jans, D.A. (1997) SV40 large tumor antigen nuclear import is regulated by the double-stranded DNA-dependent protein kinase site (serine 120) flanking the nuclear localization sequence. *J. Biol. Chem.* **272**, 22191–22198
10. Xiao, C.Y., Jans, P., and Jans, D.A. (1998) Negative charge at the protein kinase CK2 site enhances recognition of the SV40 large T-antigen NLS by importin: effect of conformation. *FEBS Lett.* **440**, 297–301
11. Jans, D.A., Xiao, C.Y., and Lam, M.H. (2000) Nuclear targeting signal recognition: a key control point in nuclear transport? *Bioessays* **22**, 532–544
12. Inoue, H., Tsuboi, Y., and Kanazawa, H. (2001) Chimeric Na(+)/H(+) antiporters constructed from NhaA of Helicobacter pylori and Escherichia coli: implications for domains of NhaA for pH sensing. *J. Biochem.* **129**, 569–576
13. Matsushita, M., Tanaka, S., Nakamura, N., Inoue, H., and Kanazawa, H. (2004) A novel kinesin-like protein, KIF1Bbeta3 is involved in the movement of lysosomes to the cell periphery in non-neuronal cells. *Traffic* **5**, 140–151
14. Kuroda, S., Tokunaga, C., Kiyohara, Y., Higuchi, O., Konishi, H., Mizuno, K., Gill, G.N., and Kikkawa, U. (1996) Protein-protein interaction of zinc finger LIM domains with protein kinase C. *J. Biol. Chem.* **271**, 31029–31032
15. Blom, N., Sicheritz-Ponten, T., Gupta, R., Gammeltoft, S., and Brunak, S. (2004) Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics* **4**, 1633–1649
16. Cassarino, D.S., Fall, C.P., Swerdlow, R.H., Smith, T.S., Halvorsen, E.M., Miller, S.W., Parks, J.P., Parker, W.D. Jr and Bennett, J.P. Jr (1997) Elevated reactive oxygen species and antioxidant enzyme activities in animal and

cellular models of Parkinson's disease. *Biochim. Biophys. Acta* **1362**, 77–86
17. Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruch, J., and Woodgett, J.R. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* **369**, 156–160
18. Robbins, D.J., Zhen, E., Cheng, M., Xu, S., Vanderbilt, C.A., Ebert, D., Garcia, C., Dang, A., and Cobb, M.H. (1993) Regulation and properties of extracellular signal-regulated protein kinases 1, 2, and 3. *J. Am. Soc. Nephrol.* **4**, 1104–1110
19. Robbins, J., Dilworth, S.M., Laskey, R.A., and Dingwall, C. (1991) Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* **64**, 615–623
20. Kogel, D., Bierbaum, H., Preuss, U., and Scheidtmann, K.H. (1999) C-terminal truncation of Dlk/ZIP kinase leads to abrogation of nuclear transport and high apoptotic activity. *Oncogene* **18**, 7212–7218
21. Cohen, O., Feinstein, E., and Kimchi, A. (1997) DAP-kinase is a Ca^{2+}/calmodulin-dependent, cytoskeletal-associated protein kinase, with cell death-inducing functions that depend on its catalytic activity. *EMBO J.* **16**, 998–1008
22. Briggs, L.J., Stein, D., Goltz, J., Corrigan, V.C., Efthymiadis, A., Hubner, S., and Jans, D.A. (1998) The cAMP-dependent protein kinase site (Ser312) enhances dorsal nuclear import through facilitating nuclear localization sequence/importin interaction. *J. Biol. Chem.* **273**, 22745–22752
23. Hubner, S., Xiao, C.Y., and Jans, D.A. (1997) The protein kinase CK2 site (Ser111/112) enhances recognition of the simian virus 40 large T-antigen nuclear localization sequence by importin. *J. Biol. Chem.* **272**, 17191–17195
24. Mancini, A., Koch, A., Whetton, A.D., and Tamura, T. (2004) The M-CSF receptor substrate and interacting protein FMIP is governed in its subcellular localization by protein kinase C-mediated phosphorylation, and thereby potentiates M-CSF-mediated differentiation. *Oncogene* **23**, 6581–6589
25. Friedrich, M.L., Cui, M., Hernandez, J.B., Weist, B.M., Andersen, H.M., Zhang, X., Huang, L., and Walsh, C.M. (2007) Modulation of DRAK2 autophosphorylation by antigen receptor signaling in primary lymphocytes. *J. Biol. Chem.* **282**, 4573–4584
26. Kuwahara, H., Kamei, J., Nakamura, N., Matsumoto, M., Inoue, H., and Kanazawa, H. (2003) The apoptosis-inducing protein kinase DRAK2 is inhibited in a calcium-dependent manner by the calcium-binding protein CHP. *J. Biochem.* **134**, 245–250
27. Nakamura, N., Miyake, Y., Matsushita, M., Tanaka, S., Inoue, H., and Kanazawa, H. (2002) KIF1Bbeta2, capable of interacting with CHP, is localized to synaptic vesicles. *J. Biochem.* **132**, 483–491
28. Matsushita, M., Sano, Y., Yokoyama, S., Takai, T., Inoue, H., Mitsui, K., Todo, K., Ohmori, H., and Kanazawa, H. (2007) Loss of calcineurin homologous protein-1 in chicken B lymphoma DT40 cells destabilizes Na+/H+ exchanger isoform-1 protein. *Am. J. Physiol. Cell Physiol.* **293**, C246–C254
29. Ohno, S. and Nishizuka, Y. (2002) Protein kinase C isotypes and their specific functions: prologue. *J. Biochem.* **132**, 509–511
30. Liu, J. and Lin, A. (2005) Role of JNK activation in apoptosis: a double-edged sword. *Cell Res.* **15**, 36–42
31. Jinlian, L., Yingbin, Z., and Chunbo, W. (2007) p38 MAPK in regulating cellular responses to ultraviolet radiation. *J. Biomed. Sci.* **14**, 303–312
32. Canning, M.T., Nay, S.L., Pena, A.V., and Yarosh, D.B. (2006) Calcineurin inhibitors reduce nuclear localization of transcription factor NFAT in UV-irradiated keratinocytes and reduce DNA repair. *J. Mol. Histol.* **37**, 285–391