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Identification of the sequence responsible for the nuclear localization of human Cdc6

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Abstract The Cdc6 is the essential protein for the initiation of DNA replication. Cdc6 is localized in the G1 nucleus, and abnormal nuclear localization of this protein induces irregular initiation of DNA replication. We identified here that amino acids K^{57} and R^{58} in the human Cdc6 protein play an important role in the nuclear localization of the protein. The fundamental features of the mechanism regulating the localization of Cdc6 seem to be maintained in yeast, Xenopus, and human, since the amino acid sequence surrounding K^{57} and R^{58}, (S/T)PXKR(L/I), is conserved in these species. Substitution of amino acid residue S^{54} with E and not Q blocked partially the nuclear localization of the protein, implying that the phosphorylation at S^{54} is involved in the regulating mechanism of the cell cycle-dependent localization of Cdc6.

Key words: Cdc6; DNA replication; Nuclear localization

1. Introduction

The recent identification of multiple eukaryotic proteins that bind directly or indirectly to origins of DNA replication has led to the search for how DNA replication is initiated and regulated in eukaryotes [1,2]. Central to this process is the origin recognition complex (ORC[1]), a tight complex of six polypeptides that bind in a sequence-specific manner to origins of DNA replication and is involved in the initiation of chromosomal DNA replication [3–5]. An additional molecule, CDC6 in *Saccharomyces cerevisiae* and cdc18 in *Schizosaccharomyces pombe*, is essential for the onset of DNA replication and is known to interact with the ORC and cdc2 kinase [3,6–11]. Recently, it is demonstrated that the central role of CDC6/cdc18 is to associate with the ORC and MCM proteins to form a pre-replication complex at the origins of DNA replication during the G1 phase [12–17]. Once replication begins, the concordant removal of CDC6/cdc18 prevents the loading of MCM proteins onto the origin-bound ORC in the G2 phase, thereby preventing rereplication of DNA. These findings point to the importance of the regulation of the concentration of CDC6/cdc18 in the nucleus for precise cell cycle-regulation.

In fungi the concentration of CDC6/cdc18 is reduced by degradation of CDC6/cdc18 as cells enter the S phase [7]. Meanwhile, in mammalian cells, it is not certain whether the Cdc6 is destroyed during the S phase as in fungi. It has recently been reported that the total level of human Cdc6 (hCdc18, $p62^{cdc6}$) [18,19] remains constant throughout the cell cycle. Human Cdc6 is localized in the G1 nucleus, while the protein is localized in the cytoplasm and not in the nucleus during the S and G2 phases [18], indicating the possibility that the concentration of Cdc6 in the nucleus is maintained by a change in the cellular localization of the protein and not by its degradation. It has been conserved from yeast to mammals that Cdc6 is not localized in the nucleus in the S phase, however; it is not yet known whether the detailed mechanism of its removal from the nucleus has also been conserved. Nuclear import of Cdc6, as well as its removal from the nucleus, are important for the precise regulation of the nuclear concentration of the protein, but the timing and mechanism have not been clarified. Tight restriction on the nuclear import of Cdc6 seems to be essential because nuclear localization of the protein at the wrong phase of the cell cycle initiates of irregular DNA replication [8–10,20–23].

The active transport of proteins between the nucleus and cytoplasm is an important process in eukaryotic cells [24,25]. The transport of proteins across the nuclear pore is generally selective and signal-dependent. The active import of a protein into the nucleus requires the presence of a nuclear localization signal (NLS) in the sequence of the imported protein, and at least four other proteins interact sequentially with the NLS-containing protein [24–26]. Several discrete steps are involved in the import process: (1) binding of the importin α (karyopherin α, PTAC58) to an NLS; (2) complex formation in conjunction with importin β (karyopherin β, PTAC97); (3) targeting of nuclear pore proteins; and (4) ATP/GTP-dependent translocation through the nuclear pore mediated by Ran. The importin α family is comprised of at least three groups of proteins: Rch1/hSRP1α, NPI-1/hSRP1, and Qip1. Many members of each of these three groups have been identified, and differential expression and the specificity of these proteins have recently been reported [27].

In the present study we investigated the nuclear translocation of human Cdc6 by transient expression of chimeric constructs of human Cdc6 and GFP. We identified the sequence within human Cdc6 that is responsible for its nuclear localization, and that this sequence can not bind to the Rch1.

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Abbreviations: BSA, bovine serum albumin; DAB, 3,3′-diaminobenzidine; DTT, dithiothreitol; EGTA, [ethylene bis(oxo-nitrilo)]tetraacetic acid; GFP, green fluorescent protein; NLS, nuclear localization signal; GST, glutathione S transferase; nt, nucleotide; ORC, origin recognition complex; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction

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2. Materials and methods

2.1. Expression of recombinant proteins

The cDNA of human Cdc6 was prepared by polymerase chain reaction (PCR) using primer set of 5′-CACTGAGGAGGTGGAAA-GAAGAGGA-3′ and 5′-CTGAGGCAAGAGAATCACTTCAACC-3′. The human leukocyte cDNA library purchased from Clontech was used as a template. This amplified cDNA was then inserted into the pGEM-T Easy vector (Promega) and the sequence of the inserted cDNA was confirmed. The cDNA inserted in the vector was used as the template for PCR to prepare the partial fragments of Cdc6 cDNA. The four partial fragments of Cdc6 generated were: nucleotide (nt) 129–1853, nt 101–555, nt 248–555, and nt 437–1599 of the cDNA sequence of human Cdc6. To express a fusion protein with GFP in HeLa cells we used the pEGFP C2 vector (Clontech). Fragment nt 129–1853 was inserted into *Xho*1-*Hin*dIII site of pEGFP C2 and nt 101–555 and nt 248–555 were inserted into *Eco*R1-*Bam*H1 site of the vector. Fragment nt 437–1599 was inserted into *Bgl*II-*Xho*1 site of the vector. To generate mutants of Cdc6, we used the QuickChange Site-Directed Mutagenesis Kit purchased from STRATAGENE, and all assays were carried out according to the manufacturer's instruction. The nt 129–1853 fragment of human Cdc6 inserted into *Xho*1-*Hin*dIII site of pEGFP C2 was used as the template. The DNA sequence of the various mutants of Cdc6 was confirmed. The partial human Cdc6 cDNA inserted in pEGFP C2 was transfected in HeLa cells using Effectene Transfection Reagent (QIAGEN). After incubation for 18 h, these cells were analyzed by confocal microscopy. Expression of these fusion proteins of GFP and the partial Cdc6 were confirmed by Western blotting using anti-GFP antibody (data not shown).

The cDNA of Rch1 was a kind gift from Dr. Ronald A. Laskey (Department of Zoology, Wellcome/CRC Institute, Cambridge, UK). The coding region, nt 133–1722, of Rch1 was amplified by PCR using appropriate primers containing a restriction enzyme site. The amplified cDNA was then inserted into the *Bam*H1-*Xho*1 site of pTrc His vector (Invitrogen) and the sequence of the inserted Rch1 cDNA inserted was confirmed. To express a fusion protein with GST in Epicurean Coli strain BL21 we used the pGEX 5X vector (Amersham Pharmacia). The cDNA of SV40 large T antigen-encoding NLS (T-NLS) was prepared by PCR of pGAD424 (Clontech) using a primer set of 5′-GCGCAGATCTCTATACCAAGCATA-CAATCAACTC-CA-3′ and CTATACCTGAGAAAGCAACCTGACCT-3′. The amplified cDNA containing T-NLS and a Gal4 activation domain was inserted into the *Bam*H1-*Eco*R1 site of pGEX 5X vector. The partial fragment of Cdc6, nt 249–555, was amplified by PCR using a pair of primers containing *Bam*H1 and *Eco*R1 restriction enzyme sites, respectively. The amplified DNA fragment was digested by *Bam*H1 and *Eco*R1 and inserted into the *Bam*H1-*Eco*R1 site of pGEX 5X vector. The Rch1 inserted in pTrc His, the partial fragment of Cdc6, nt 249–555, inserted in pGEX 5X and the T-NLS inserted in pGEX 5X were each transfected individually into the BL21 strain, respectively. After induction with 0.1 mM IPTG at 37°C for 4 h each strain was harvested and lysed by sonication in the 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 1 mM EDTA and protease inhibitors. Each lysate was centrifuged at $30\,000\times g$ for 30 min. The supernatants were obtained as recombinant protein fractions.

2.2. Precipitation assay

The GST partial human Cdc6 fusion protein and the GST-T-NLS fusion protein were each incubated with or without His_6-tagged Rch1 in a solution of 0.05% BSA, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, and 1 mM EDTA and incubated at 4°C for 15 min. The GST fusion proteins were selectively precipitated by glutathione Sepharose 4B (Amersham Pharmacia). The precipitate was washed four times with 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, and 1 mM EDTA. The proteins precipitated with glutathione Sepharose 4B were eluted by a solution which consisted of 20 mM glutathione, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, and 1 mM EDTA, and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins separated by SDS-PAGE were transferred to a PVDF membrane at a constant voltage of 15 V for 1 h. The membrane was blocked with 5% non-fat milk in PBS and probed with anti-His_6 antibody in 5% non-fat milk in PBS. The bound antibodies were detected with a peroxidase-conjugated secondary antibody (Amersham Pharmacia) at 1:500 dilution in 5% non-fat milk in PBS, and the protein bands were visualized by the colorimetric method with DAB as the substrate.

3. Results and discussion

3.1. N-terminal portion of human Cdc6 is required for nuclear localization

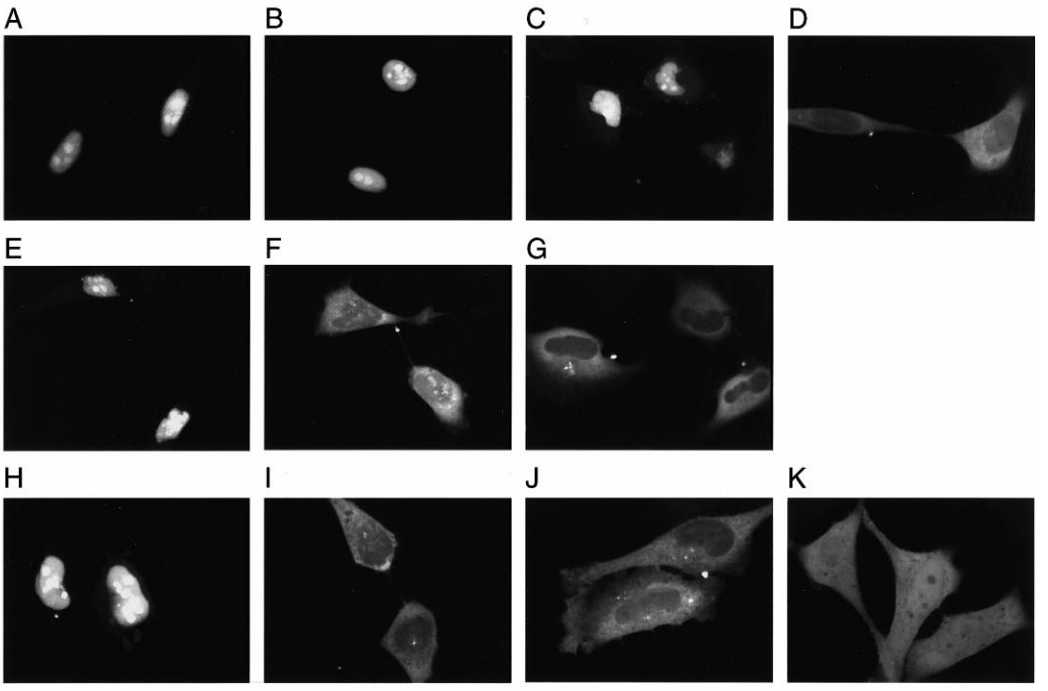
To determine the subcellular localization of human Cdc6, the fusion protein of GFP and Cdc6 was expressed in HeLa cells. The fluorescence of the GFP-Cdc6 fusion protein was clearly localized in the nucleus, and the protein was clustered in a part of the nucleus (Fig. 1A). We observed the nuclear localization of GFP-tagged human Cdc6 in more than 90% of the cells expressing the fusion protein in separate experiments. Deletion of the C-terminal portion of Cdc6 showed no change in the nuclear localization of Cdc6 (Fig. 1B and C). The fusion protein of GFP and the C-terminal portion of Cdc6 was localized only in the cytoplasm (Fig. 1D). These results demonstrate that the N-terminal portion of Cdc6, nt 249–555 of Cdc6 plays an important role in the nuclear localization of the protein. These results are consistent with a previous report which points out the importance of the N-terminal portion of *S. cerevisiae* CDC6 for the nuclear localization of this protein [28].

The NLSs included in many karyopheric proteins contain clusters of basic amino acid residues [29]. The N-terminal portion of Cdc6 encoded by the nt 249–555 contains three basic amino acid clusters, amino acids 16–18, 56–58 and 92–95, as shown in Fig. 1L. Saha et al. [18], reported that amino acids 92–95 make no contribution to nuclear localization. We also could not detect the contribution of this cluster to the nuclear localization of Cdc6 (Fig. 1D) and we examined the other clusters. We found that the nuclear localization of Cdc6 mainly depends on amino acids 56–58, RKR. As shown in Fig. 1E and F, the Cdc6 mutant whose amino acids KRK, 16–18, were changed to NCN was localized in the nucleus, while the Cdc6 mutant whose amino acids RKR, 56–58, were changed to SNG was localized in the cytoplasm. Mutant Cdc6 which included mutations in both amino acid clusters, 16–18 and 56–58, was also localized in the cytoplasm (Fig. 1G). These results demonstrate that the nuclear localization of human Cdc6 is directed by the basic amino acid residues, ^{56}RKR^{58}.

To determine whether all three amino acids, RKR, are essential for the nuclear localization of Cdc6, we prepared fragments of Cdc6 cDNA which result in a single amino acid mutation in this region. The Cdc6 mutant with $R^{56}S$ was able to localize to the nucleus (Fig. 1H). The mutant which includes either $K^{57}N$ or $R^{58}G$ was not localized in the nucleus (Fig. 1I and J), which indicates that both K^{57} and R^{58} are essential amino acids involved in the nuclear localization of human Cdc6. These results suggest that the NLS within the human Cdc6 protein is comprised of amino acid residues near K^{57} and R^{58}.

3.2. N-terminal portion of Cdc6 can not bind to Rch1

Since the first step of protein import from the cytosol to the nucleus is the binding of importin α to the NLS of karyopheric protein in the cytosol [24–26], we next assessed the binding of Rch1, one of the human importin α, to the N-terminal portion of human Cdc6. The NLSs of various kar-



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249            ttttccaaaaaggaagctgtctcgggcattgaacaaagctaaaaactccagt
14               F  P  K  R  K  L  S  R  A  L  N  K  A  K  N  S  S

301    gatgccaaactagaaccaacaaatgtccaaaccgtaacctgttctcctcgtgtaaaagcc
31      D  A  K  L  E  P  T  N  V  Q  T  V  T  C  S  P  R  V  K  A

361    ctgcctctcagccccaggaaacgtctgggcgatgacaacctatgcaacactccccattta
51      L  P  L  S  P  R  K  R  L  G  D  D  N  L  C  N  T  P  H  L

421    cctccttgttctccaccaaagcaaggcaagaaagagaatggtccccctcactcacataca
71      P  P  C  S  P  P  K  Q  G  K  K  E  N  G  P  P  H  S  H  T

481    cttaagggacgaagattggtatttgacaatcagctgacaattaagtctcctagcaaaaga
91      L  K  G  R  R  L  V  F  D  N  Q  L  T  I  K  S  P  S  K  R

541    gaactagccaaagtt
111     E  L  A  K  V
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Fig. 1. Cellular localization of partial human Cdc6. A–D: Partial fragments of human Cdc6 cDNA were inserted into the pEGFP C2 vector, and transfected to HeLa cells. After 18 h, the cells were analyzed by confocal microscopy to observe the localization of the fusion protein of GFP and partial human Cdc6. The four partial fragments of the cDNA of human Cdc6 inserted into pEGFP C2 were: (A) nt 129–1853, (B) nt 101–555, (C) nt 249–555, and (D) nt 437–1599. E–J: Fragments of human Cdc6 cDNA, nt 129–1853, containing various mutations, were inserted into the pEGFP C2 vector and transfected to HeLa cells. After 18 h, the cells were analyzed by confocal microscopy to observe the localization of the fusion protein of GFP and mutant human Cdc6. The localization of mutant human Cdc6 with (E) amino acids KRK, 16–18, substituted with NCN, (F) amino acids RKR, 56–58, substituted with SNG, (G) amino acids KRK, 16–18 with NCN and 56–58, RKR with SNG, (H) $R^{56}S$, (I) $K^{57}N$, and (J) $R^{58}G$, are shown. K: pEGFP C2 vector which is not inserted into any DNA fragment was transfected to the HeLa cells as a control. L: The N-terminal portion of the cDNA sequence of human Cdc6, nt 249–555, and the encoded amino acids. The clusters of basic amino acid residues are indicated in italic.

yopheric proteins thus far identified can be classified into two major classes: (1) a single cluster of basic amino acids such as in T-NLS, and (2) the bipartite type, in which two sets of adjacent basic amino acids are separated by a stretch of approximately 10 amino acids [29]. It has been demonstrated that each of the two types of NLS is the binding site of importin α [30]. As shown in Fig. 2, the His_6-Rch1 did not co-precipitate with GST-Cdc6, while the protein co-precipitated with the fusion protein of GST and T-NLS, indicating that the nuclear localization of Cdc6 is not regulated by the directly binding of Rch1. Recently, many members of importin α family have been identified, and differential expression and the specificity of these proteins have recently been reported [27]. The Cdc6 may use other importin α family pro-

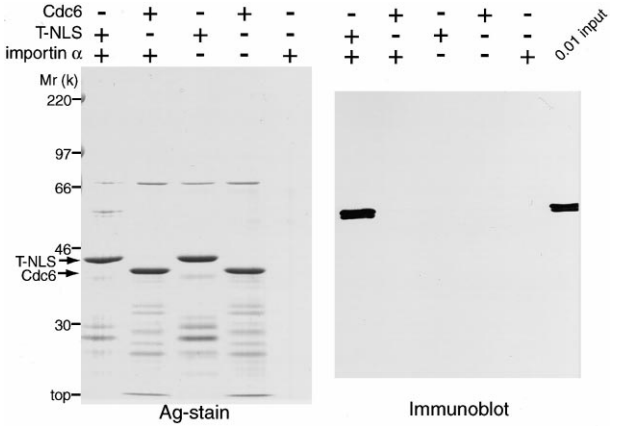

Fig. 2. Immunoprecipitation assay of human Cdc6 and T-NLS. The GST partial human Cdc6 fusion protein and the GST-T-NLS fusion protein were each incubated with or without His_6-tagged Rch1. After 15 min, the GST fusion proteins were selectively precipitated by glutathione Sepharose 4B and analyzed by SDS-PAGE. The proteins separated by SDS-PAGE were detected by Ag staining or transferred to a PVDF membrane. The membrane was probed with anti-His_6 antibody. The bound antibodies were detected with a peroxidase-conjugated secondary antibody and the protein bands were visualized by the colorimetric method with DAB as the substrate. In lane '0.01 input', the 0.01 volume of His_6-tagged Rch1 fraction was analyzed by SDS-PAGE and transferred to a PVDF membrane.

tein. Since we could not classify the sequence near K^{57} and R^{58} within human Cdc6 into either the single or bipartite type NLS group, another possibility is that the import of Cdc6 is dependent on binding with another karyopheric protein which can bind to an importin α family protein.

3.3. The Cdc6 mutant with $S^{54}E$ localized in both the cytosol and the nucleus

Alignment of the amino acid sequence of CDC6, cdc18, Xenopus Cdc6, and human Cdc6 is shown in Fig. 3. The amino acid residues, (S/T)PXK^{57}R^{58}(L/I), are conserved among these species, although the N-terminal portions revealed low homology, implying that the mechanism of the translocation of Cdc6 from the cytosol to the nucleus has been conserved in these species.

Since the conserved sequence, (S/T)PXK^{57}R^{58}(L/I), includes a putative cdc2 kinase phosphorylation site and mutagenesis at K^{57} disrupts the consensus sequence of the phosphorylation site, we examined whether a phosphorylation of S^{54} in the conserved sequence is necessary for the nuclear localization of Cdc6. The Cdc6 mutant with $S^{54}Q$ was able to localize to the nucleus in a manner similar to the wild-type Cdc6 (Fig. 4A and B), which indicates that phosphorylation of S^{54} is not

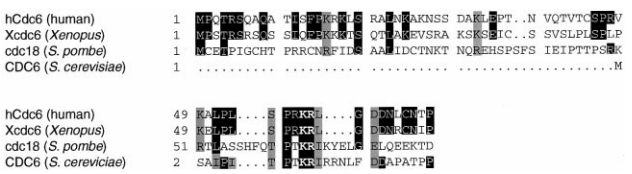

Fig. 3. Multiple alignment of the N-terminal portion of human Cdc6, *Xenopus laevis* Cdc6, *S. pombe* cdc18 and *S. cerevisiae* CDC6. Identical amino acid residues are indicated by dark shading, and conservative substitutions are indicated by light shading. The $K^{57}R^{58}$ are indicated in bold.

necessary for the nuclear localization of Cdc6. The Cdc6 mutant with $S^{54}E$ was shown to be localized in both the nucleus and the cytoplasm (Fig. 4C). A cluster of fusion proteins was not observed (Fig. 4C), whereas the cluster was clearly observed in the nucleus when the wild-type and mutant with $S^{54}Q$ were localized in the nucleus (Fig. 1A–C, E, H and Fig. 4A and B). These results suggest that a negative charge at S^{54} within human Cdc6 plays an inhibitory role in the nuclear and intra-nuclear localization of Cdc6. Since the amino acid residue S^{54} is a putative cdc2 kinase phosphorylation site, these results suggest the possibility that the localization of Cdc6 is regulated by the phosphorylation and are consistent with the findings that the Cyclin A/Cdk2-mediated phosphorylation of Cdc6 inhibits the nuclear localization of the protein [31].

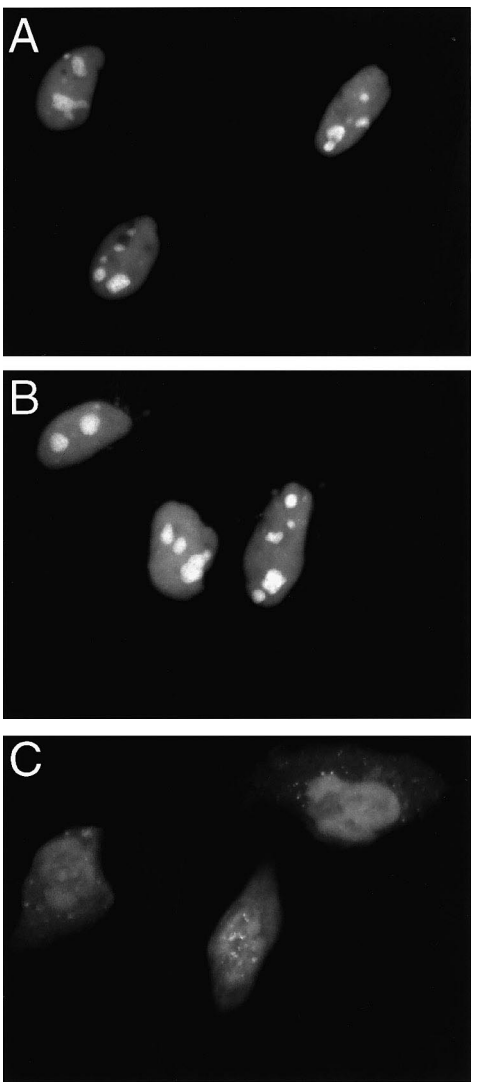

Fig. 4. Effect of an amino acid substitution at amino acid residue S^{54} within human Cdc6 on the nuclear localization of the protein. Fragments of human Cdc6 cDNA, nt 129–1853, containing various mutations, were constructed and transfected to HeLa cells. After 18 h, the cells were analyzed by confocal microscopy to observe the localization of the fusion protein of GFP and mutant human Cdc6. The localizations of mutant human Cdc6 with (A) wild-type Cdc6, (B) mutant human Cdc6 with amino acid S^{54} substituted with Q, (C) mutant human Cdc6 with amino acid S^{54} substituted with E, are shown.

In summary, we demonstrated that the amino acids K^{57} and R^{58} within the human Cdc6 protein play an important role in the nuclear localization of the protein and the amino acid sequence near K^{57} and R^{58}, (S/T)PX$K^{57}R^{58}$(L/I), is conserved in fungi, Xenopus, and human. We also demonstrated that the partial fragment of Cdc6 which is sufficient for the nuclear localization can not bind to Rch1. We suggest that the mechanism regulating the localization of Cdc6 may include the phosphorylation of amino acid S^{54} which is one of a putative cdc2 kinase phosphorylation site within the Cdc6. To study the timing of the formation of the pre-replication complex, the mechanism which restricts the nuclear localization of Cdc6 must be clarified since nuclear localization of the protein triggers the initiation of DNA replication [8–10,20–23]. Our data presented here are useful for the study of the cell cycle-regulated nuclear localization mechanism of Cdc6. Further investigation will clarify the detail translocation mechanism of the human Cdc6 into the nucleus.

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