Biochemical and Biophysical Research Communications **274,** 37–42 (2000)
doi:10.1006/bbrc.2000.3098, available online at http://www.idealibrary.com on IDEAL®

Minimal Requirements for the Nuclear Localization of p27^{Kip1}, a Cyclin-Dependent Kinase Inhibitor

Ying Zeng, Katsuya Hirano, Mayumi Hirano, Junji Nishimura, and Hideo Kanaide[1]
Department of Molecular Cardiology, Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Received June 6, 2000

p27^{Kip1} is a cyclin-dependent kinase inhibitor, and its nuclear localization is a prerequisite for it to function as a cell cycle regulator. In the present study, the minimal requirement for the nuclear localization signal (NLS) of p27^{Kip1} was determined by analyzing the localization of various mutants of p27^{Kip1} tagged with green fluorescent protein (GFP) in HeLa cells and porcine aortic endothelial cells. Wild-type p27^{Kip1} exclusively localized into nucleus, while GFP alone localized in both cytosol and nucleus. A comparison of various truncation mutants revealed residues 153–166 to be the minimal region necessary for nuclear localization. However, a fusion of this region to GFP showed cytoplasmic retention in addition to nuclear localization, thus suggesting that some extension flanking this region is required to achieve a full function of NLS. The site-directed mutation of the full-length p27^{Kip1} therefore showed that four basic residues (K153, R154, K165, R166), especially R166, play a critical role in the nuclear localization of p27^{Kip1}.

The proliferation of mammalian cells is primarily regulated during the prereplicative (G_1) phase of the cell cycle (1, 2). The basic components of the machinery involved in the regulation of the cell cycle are cyclins and cyclin-dependent kinases (Cdks) which form active kinase complexes, and Cdk inhibitors (3, 4). Two classes of Cdk inhibitors have been identified: The INK4 family that are specific inhibitors of cyclinD-Cdks, and the Kip/Cip family including p21, p27, and p57, which inhibit all types of cyclin-Cdk complexes (5, 6). Nuclear localization is a prerequisite for these proteins to function as cell cycle regulators. p27^{Kip1} is considered to play a critical role in the growth arrest induced either by cell-cell contact or serum deprivation (7, 8). Recent studies have suggested that the nuclear localization of p27^{Kip1} closely correlates with its ability to inhibit cell cycle progression (9–13).

The nuclear pore complexes (NPCs) provide channels which measure about 9 nm in diameter, and thus allow the diffusion of ions, metabolites and small proteins (relative molecular mass less than 40–60 kDa), mediate the selective transport of particles up to 26–28 nm in diameter by energy-dependent mechanisms and serve as sites of exchange of macromolecules between cytoplasm and nucleus in eukaryotic cells (14, 15). Many, if not all, of the nuclear proteins contain a nuclear localization signal (NLS), which promote the active transport into nucleus. Classical NLSs are short sequences containing several essential basic amino acids. When fused to a heterologous protein, these short NLS sequences are sufficient to direct the chimerical polypeptide into the nucleus (16). There are two major types of NLS: (1) a monopartite NLS, composed of a single cluster of basic amino acids, (2) a bipartite NLS, composed of two clusters of basic amino acids separated by a spacer region composed of non-basic amino acids. SV40 large T antigen contains a typical example of a monopartite NLS (17), and nucleoplasmin contains a typical bipartite NLS (18). p27^{Kip1} has been suggested to contain a putative bipartite NLS at the C-terminal region, which consists of an N-terminal cluster of 3 basic residues and a C-terminal cluster of two basic residues (Fig. 3) (19). However, the function of this region 152–166 as an NLS has not yet been proven, and the minimal requirement of amino acid residues for the nuclear localization of p27^{Kip1} has not yet been determined.

In the present study, we determined the region required for the nuclear localization of p27^{Kip1} and the amino acid residues essential for NLS, by examining the subcellular localization of p27^{Kip1} and its various truncated and site-directed mutants which were expressed as fusion proteins with green fluorescence protein (GFP). We demonstrated that a specific region, 153–166, serves as a functional NLS in p27^{Kip1} and that four out of five basic residues are essential to achieve its function.

[1] To whom correspondence should be addressed. Fax: +81-92-642-5552. E-mail: kanaide@molcar.med.kyushu-u.ac.jp.

0006-291X/00 $35.00

TABLE 1

List of PCR Primers for Wild-Type $p27^{Kip1}$ (Kip1) and Its Truncated Mutants

	Upper primers	Lower primers
Kip1		5′ Acg AgC Agg ATC CgT TTg ACg CCT TCT gAg 3′
Kip1(1–166)	5′ CTT gCg ggg TTT TTC AgT A 3′	5′ TTC TgT Tgg ATC CgC TCT TTT gTT TTg Agg 3′
Kip1(1–165)		5′ TCT gTT ggg ATC CTT gTT TTg Agg AgA ggA 3′
Kip1(152–198)	5′ CAg TgA ATT Cgg ATg Agg AAg CgA CCT gCC 3′	
Kip1(153–198)	5′ CAg TgA ATT Cgg ATA ATg AAg CgA CCT gCC 3′	5′ ACg AgC Agg ATC CgT TTg ACg CCT TCT gAg 3′
Kip1(154–198)	5′ ACT ggA ATT Cgg ATg CgA CCT gCC ACA gAC 3′	
Kip1(155–198)	5′ ggg AgA ATT CAg ATg CCT gCC ACA gAC gAT 3′	
Kip1(152–166)	5′ CAg TgA ATT Cgg ATg Agg AAG CgA CCT gCC 3′	5′ TTC TgT Tgg ATC CgC TCT TTT gTT TTg Agg 3′

Note. Eco RI sites (upper primers) and *Bam* HI sites (lower primers) are underlined. The initiation condons integrated in the upper primers are double-underlined.

METHODS

Cell culture. HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin. Porcine aortic endothelial cells were obtained as previously described (20), and cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 μg/ml gentamicin.

Plasmid construction. A clone for porcine $p27^{Kip1}$ (Accession No. AB031955) was obtained through the screening of a cDNA library constructed from a contact-induced growth arrested endothelial cells (21). The insert containing a 5′ untranslated region and a full coding region of the clone (pAD-Kip1) was obtained by polymerase chain reaction (PCR), digested with *Eco*RI and *Bam*HI, and ligated to *Eco*RI and *Bam*HI sites of pEGFP-N1 (Clontech, Palo Alto, CA). The PCR reaction utilized an upper primer corresponding to the vector of the cDNA library (pAD, Stratagene, La Jolla, CA), and a lower primer corresponding to a region containing a termination codon (Table 1). In the lower primer, the termination codon was destroyed and the *Bam*HI site was created. An *Eco*RI site of the PCR product was a cloning site of the library. The expression plasmid (pEGFP-Kip1) thus obtained was used to express a wild type full-length $p27^{Kip1}$ (referred to as Kip1) which fused to the N-terminus of GFP.

All cDNAs for the truncation mutants of $p27^{Kip1}$ were obtained by PCR using the primer sets listed in Table 1 and pAD-Kip1 as template. The upper primers for the N-terminal truncation were designed to contain *Eco*RI site for ligation to the vector and an initiation codon. The lower primers were designed to contain a *Bam*HI site. The truncation mutants thus obtained were Kip1(1–166), encoding residues 1–166, Kip1(1–165), Kip1(152–198), Kip1(153–198), Kip1(154–198) Kip1(155–198), and Kip1(152–166). We introduced the site-directed mutation to Kip1 using overlap extension PCR technique (22). Complementary forward and reverse primers containing mutations (Table 2) and the upper and lower primers corresponding to the regions of pEGFP-N1 flanking the cloning sites (see the legend for Table 2) were used to generate two DNA fragments having overlapping ends by PCR amplification using 100 ng pEGFP-Kip1 as template. The two PCR products were purified through agarose gel electrophoresis, and subjected to the extension PCR using the upper and lower primers corresponding to pEGFP-N1. The product of the extension PCR was digested with *Eco*RI and *Bam*HI, and then ligated to *Eco*RI and *Bam*HI site of pEGFP-N1. The site-directed mutations thus obtained were Kip1(R152A) (mutation of R152 to A), Kip1(K153A), Kip1(R154A), Kip1(K165A), and Kip1(R166A). Plasmid DNA was purified with plasmid purification Kits (Qiagen, Hilden, Germany) for transfection, according to the procedure specified by the manufacturer.

The DNA sequences were determined by the dideoxy-mediated chain termination method on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA). We confirmed that all constructs contained no un-intended mutation.

Transfection of cells with expression plasmid. HeLa cells were transfected with the expression plasmid using LipofectAMINE (Life Technologies, Rockville, MD). Forty thousand cells were seeded on coverslips in 35-mm tissue culture dish on the day before transfection. After rinsing with serum-free, antibiotics-free DMEM, the cells were transfected by incubation in 1 ml serum-free, antibiotics-free DMEM containing 5 μl LipofectAMINE and 2–3 μg plasmid DNA at 37°C for 5 h. LipofectAMINE and plasmid DNA had been incubated at room temperature for 15 min before transfection. After 5 h incubation, the transfection mixture was replaced with 10% serum containing complete growth medium, and cells were cultured for 16–24 h.

TABLE 2

List of Complementary Primers Containing Site-Directed Mutations for the Full-Length $p27^{Kip1}$

	Forward primers	Reverse primers
Kip1(R152A)	5′ TgC ACT ggg ATA gCg AAg CgA CCT gCC 3′	5′ ggC Agg TCg CTT CgC TAT CCC AgT gCA 3′
Kip1(K153A)	5′ C ACT ggg ATA Agg gCg CgA CCT gCC ACA g 3′	5′ C TgT ggC Agg TCg CgC CCT TAT CCC AgT g 3′
Kip1(R154A)	5′ C ACT ggg ATA Agg AAg gCA CCT gCC ACA gAC g 3′	5′ C gTC TgT ggC Agg TgC CTT CCT TAT CCC AgT g 3′
Kip1(K165A)	5′ AT TCC TCT CCT CAA AAC gCA AgA gCC AAC AgA ACA gA 3′	5′ TC TgT TCT gTT ggC TCT TgC gTT TTg Agg AgA ggA AT 3′
Kip1(R166A)	5′ AT TCC TCT CCT CAA AAC AAA gCA gCC AAC AgA ACA gA 3′	5′ TC TgT TCT gTT ggC TgC TTT gTT TTg Agg AgA ggA AT 3′

Note. The primers listed above were utilized to generate the two DNA fragments having overlapping ends in the initial polymerase chain reaction. One product was obtained by a combination of the upper primer (5′ gCA gAg CTg gTT TAg TgA AC 3′) located upstream to the *Eco* RI cloning site of pEGFP-Kip1 and the reverse primers as listed; the other product was obtained by a combination of the forward primers as listed and the lower primer (5′ ACg AgC Agg ATC CgT TTg ACg CCT TCT gAg 3′) located downstream to the *Bam* HI cloning site of pEGFP-Kip1. Codons containing mutant are underlined.

The transfected cells were then fixed in 2% paraformaldehyde at room temperature for 5 min. After washing three times with phosphate buffered saline, coverslips were mounted on glass slides and sealed with nail oil.

Confocal fluorescence microscopy. The fluorescence images were observed under a laser scanning microscopy LSM GB200 (Olympus, Tokyo, Japan), using a 60 × objective lens, 488 nm laser excitation, and a 500–530 band pass emission filter. Fluorescence images were obtained at the nuclear level, and saved as TIFF files for representative photos and quantitative analyses.

Quantification of fluorescence ratio of cytoplasm/nucleus. The fluorescence intensity for a certain area of cytoplasm and nucleus in the same cell was determined on a fluorescence image obtained with a confocal microscope using the NIH Image 1.62 program, and then a fluorescence ratio of cytoplasm to nucleus was obtained for each cell. The average fluorescence ratio was obtained from at least 10 different cells for each construction.

Statistical analysis. Statistical significance was evaluated by Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

A Region of p27^{Kip1} Required for Nuclear Localization

To delineate the region required for the nuclear localization of p27^{Kip1}, we examined the localization of various truncation mutants of p27^{Kip1} tagged with GFP at a steady state after transfection in a human epithelial cell-derived cell line, HeLa cells (Fig. 1), and porcine aortic endothelial cells (Fig. 2). The amino acid sequence of the porcine p27^{Kip1} is 91.9% identical to its human counterpart. Approximately 40–50% of all HeLa cells showed GFP fluorescence 16–24 h after transient transfection, while approximately 2–5% of the endothelial cells showed GFP fluorescence. Similar results for the transfection of p27^{Kip1} and its mutants were obtained in HeLa cells and endothelial cells.

The GFP tag alone (27 kDa) distributed in both cytoplasm and nucleus, with a slightly denser accumulation in nucleus, while Kip1 (22 kDa + 27 kDa tag), a wild-type p27^{Kip1} tagged with GFP, exclusively localized to nucleus (Figs. 1A and 2A). The deletion of the C-terminal region 167–198, Kip1(1–166), caused localization mostly in nucleus but with a slight degree of cytosolic fluorescence. A further deletion of only one amino acid from the C-terminus of Kip1(1–166), Kip1(1–165), caused a fluorescence pattern similar to that seen with GFP alone. On the other hand, a deletion of the N-terminal region 1–151, Kip1(152–198), had no effect on the localization of Kip1, and it showed exclusive nuclear localization. One basic amino acid (R152) deletion, Kip1(153–198), caused a slight retention of fluorescence in cytoplasm, but most of the fusion protein localized to the nucleus. However, one more basic amino acid deletion, Kip1(154–198), caused a fluorescence pattern similar to that observed with GFP alone. Kip1(155–198), which missed the whole upstream cluster of basic amino acids of a putative NLS, also showed a fluorescence pattern similar to that observed with GFP alone.

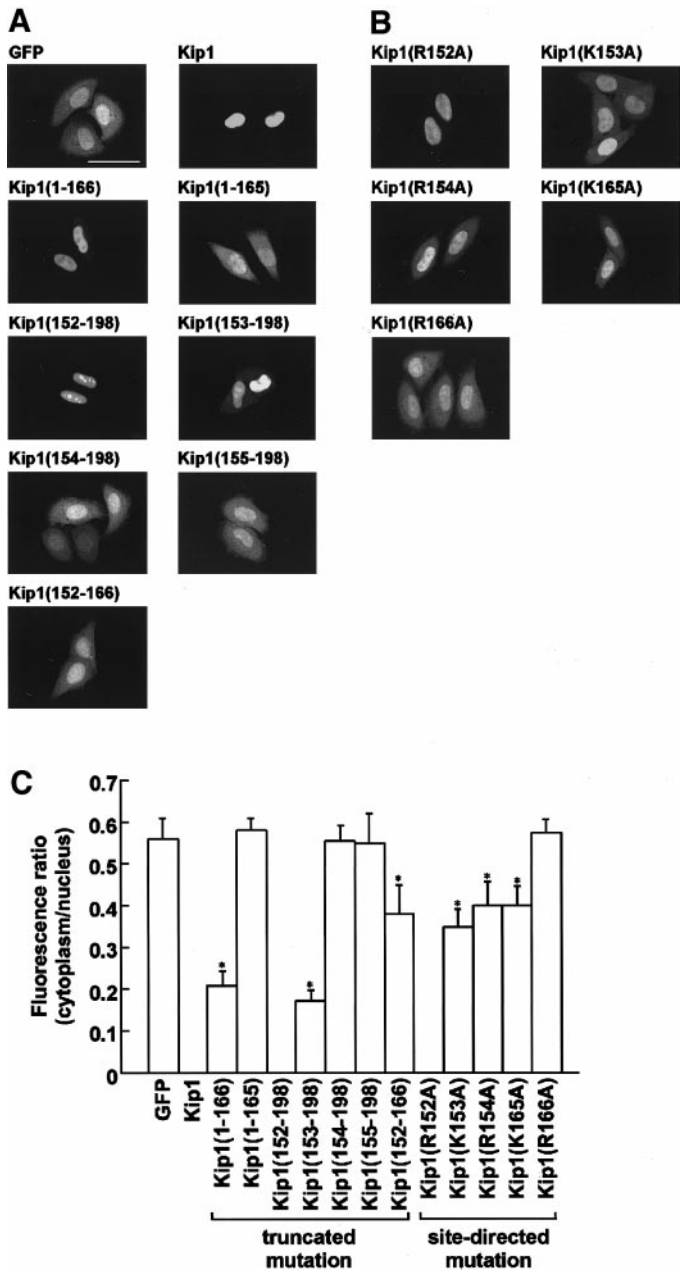

FIG. 1. Localization of p27^{Kip1} and its mutants in HeLa cells. (A and B) Representative photos of a confocal image showing GFP fluorescence of truncated (A) and site-directed mutants (B). GFP, cells expressing a GFP tag alone; Kip1, wild type p27^{Kip1}; Kip1(1–166), a truncation mutant containing residues 1–166; Kip1(1–165), a truncation mutant containing residues 1–165; Kip1(152–198), a truncation mutant containing residues 152–198; Kip1(153–198), a truncation mutant containing residues 153–198; Kip1(154–198), a truncation mutant containing residues 154–198; Kip1(155–198), a truncation mutant containing residues 155–198. Kip1(R152A), Kip1(K153A), Kip1(R154A), Kip1(K165A), and Kip1(R166A), a full-length p27^{Kip1} containing a site-directed mutation of R152, K153, R154, K165, and R166 to A, respectively. Scale bar, 50 μm. (C) A quantitative evaluation of cytoplasmic and nuclear localization. The ratio of the cytoplasmic fluorescence intensity to the nuclear fluorescence intensity was obtained for each construct. The data are the mean ± s.e. mean of 10 different cells. *, $P < 0.01$ compared with the GFP tag alone.

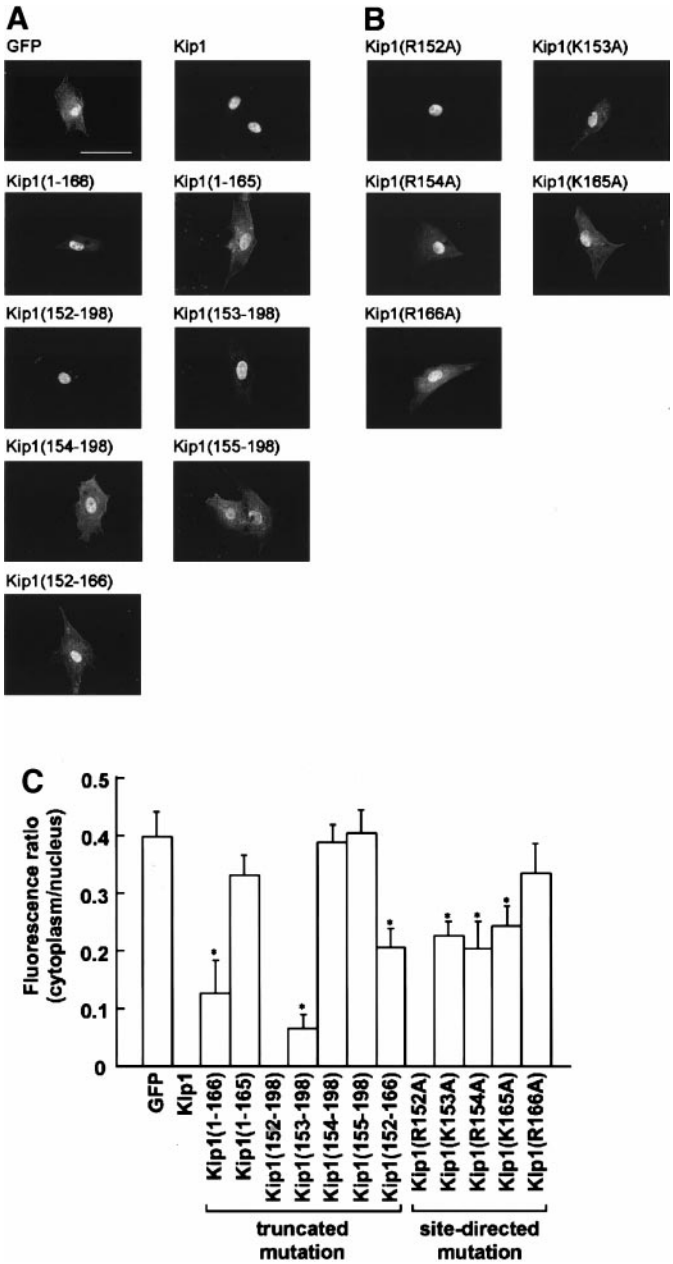

FIG. 2. Localization of $p27^{Kip1}$ and its mutants in porcine endothelial cells. (A and B) Representative photos of a confocal image showing GFP fluorescence of truncated (A) and site-directed mutants (B). The headings for photos are same as in Figs. 2A and 2B. Scale bar, 50 μm. (C) Quantitative evaluation of cytoplasmic and nuclear localization. The ratio of the cytoplasmic fluorescence intensity to the nuclear fluorescence intensity was obtained for each construct. The data are the mean ± s.e. mean of 10 different cells. *, $P < 0.01$ compared with the GFP tag alone.

The subcellular localization between cytosol and nucleus in HeLa cells (Fig. 1C) and endothelial cells (Fig. 2C) was quantitatively evaluated by determining the fluorescence ratio of cytoplasm to nucleus on a confocal microscopic image using the NIH Image 1.62 program. The fluorescence ratio for GFP was 0.56 ± 0.05 in HeLa cells and 0.40 ± 0.04 in endothelial cells. The values for Kip1 and Kip1(152–198) were 0, since no fluorescence was detected in the cytoplasm. The fluorescence ratio for Kip1(153–198) (0.17 ± 0.03 in HeLa cells and 0.06 ± 0.02 in endothelial cells) and Kip1(1–166) (0.21 ± 0.03 in HeLa cells and 0.13 ± 0.05 in endothelial cells) was higher than 0, but significantly lower than that obtained with GFP alone, thus indicating the efficiency of NLS to localize protein into nucleus was slightly attenuated. The fluorescence ratio for Kip1(154–198) (0.56 ± 0.04 in HeLa cells and 0.39 ± 0.03 in endothelial cells), Kip1(155–198) (0.55 ± 0.07 in HeLa cells and 0.40 ± 0.04 in endothelial cells) did not differ significantly from that obtained with GFP alone, indicating complete loss of NLS function. As a result, the residues 153–166 (KRPATDDSSPQNKR) included in a putative NLS was demonstrated to be necessary for the nuclear localization of $p27^{Kip1}$. However, a construct containing only this region, Kip1(152–166), was highly distributed in cytoplasm, but the fluorescence ratio (0.38 ± 0.07 in HeLa cells and 0.21 ± 0.03 in endothelial cells) was significantly lower than that obtained with GFP alone. This finding indicated that this region indeed functioned as a NLS, but its efficiency was greatly impaired.

Minimal Requirement of Amino Acids for the Nuclear Localization of $p27^{Kip1}$

To identify the essential amino acid residues for the NLS, site-directed mutagenesis in the full-length $p27^{Kip1}$ was utilized. Five basic amino acids (R152, K153, R154, K165, and R166) were substituted with A one by one (Figs. 1C and 2C). Mutation of R152 to A, Kip1(R152A) exclusively localized to nucleus as observed with a wild-type $p27^{Kip1}$. This observation was consistent with the results obtained with Kip1(153–198), thus suggesting that R152 is unnecessary for nuclear localization. However, other mutations greatly impaired the nuclear localization. Kip1(R166A) completely lost nuclear localization, and its fluorescence ratio (cytosol/nucleus) did not significantly differ from that obtained with GFP. Kip1(K153A), Kip1(R154A), and Kip1(K165A) substantially attenuated the nuclear localization, although the fluorescence ratio was slightly but significantly lower than that obtained with GFP.

DISCUSSION

We herein present the first direct evidence that a putative NLS, 152–166, serves as a functional NLS in $p27^{Kip1}$. This region (RKRPATDDSSPQNKR) contains a cluster of three basic residues at the N-terminus and a cluster of two basic residues at the C-terminus. However, a comparison of the truncated mutants delineated the region 153–166 to be the minimal sequence necessary for nuclear localization. Among the five basic

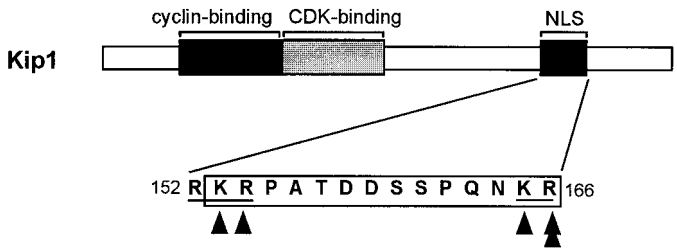

FIG. 3. The minimal sequence required for nuclear localization of $p27^{Kip1}$. A structure of Kip1 is schematically shown, and a putative nuclear localization signal (residues 152–166) and its amino acid sequence are shown. The basic amino acids residues are underlined. The minimal sequence (residues 153–166) required for nuclear localization, which was proved in the present study, is framed. The essential basic amino acids are indicated by arrowheads, with R166 being indicated by a double arrowhead to emphasize its greatest contribution to nuclear localization.

residues seen in a putative NLS, we demonstrated that K153, R154, K165, and R166 play a critical role in nuclear localization. We thus concluded the region 153–166 to be the minimal region required for the nuclear localization of $p27^{Kip1}$, while the two basic residues at both ends separated by a spacer region of 10 non-basic amino acids are the essential amino acids for NLS (Fig. 3).

The NLS-dependent nuclear import requires the formation of a NPC-targeting complex, which is composed of two nuclear targeting proteins, importin α and importin β (18). Importin α recognizes the classical NLS and binds to importin β via an importin β-binding domain. As a result, it functions as an adapter molecule between the NLS-containing protein and importin β. Importin β then targets the complex to NPC. The crystallographic analysis of importin α complexed with a peptide containing classic NLS showed that the binding of NLS occurs at two sites (23). The smaller binding site allows for the specific recognition of two basic (K or R) residues, while the larger binding site is structurally optimal for the recognition of five basic residues. The smaller binding site must be located upstream of the larger binding site, with the optimal distance being 10 residues. This observation is consistent with the consensus model for bipartite NLS (16): B(2)-N(10–12)-B(3–4 out of 5 residues), where B is a basic amino acid and N is any amino acid. The functional NLS in $p27^{Kip1}$ is thus consistent with this consensus sequence. It should be noted that this binding model indicated by the crystallographic study correlated well with the minimal requirement of two basic residues at the N-terminus as proved in the present study, but not with a requirement of three basic residues as suggested by a putative NLS.

In the present study, we quantitatively evaluated the subcellular localization by determining the mean fluorescence density for a certain area using an image analysis software package. Since a confocal fluorescence image was a slice with a certain depth, the mean fluorescence intensity for a certain area reflects the mean concentration of GFP protein. Therefore, the ratio of cytosolic to nuclear amount can be estimated by the fluorescence ratio. Based on this method, the efficiency of NLS could thus be statistically evaluated. For example, three truncation mutants, Kip1(1–166), Kip1(153–198), Kip1(152–166) and three site-directed mutants, Kip1(K153A), Kip1(R154A), Kip1(K165A) showed a significantly higher degree of nuclear staining than GFP alone, while Kip1(R166A) completely lost its NLS function. We thus concluded that the region 153–166, was necessary for nuclear localization, but this region alone was insufficient to achieve the full efficiency of NLS. Some extension flanking the minimal NLS are thus suggested to be required for the full function of NLS. A similar requirement of such extension has been noted for the interaction between the NLS of the helicase Q1 and Qip1, a member of the NLS receptor family (24). Based on the findings of a quantitative analysis, we also concluded that R166 is the most important basic residue among the four essential basic amino acids.

As we demonstrated in the present study, the NLS plays an essential role in determining the subcellular localization of $p27^{Kip1}$. However, the localization of $p27^{Kip1}$ and also its function as a negative regulator of cell cycle could also be regulated by other factors. Orend *et al.* (10) found $p21^{Cip1}$ and $p27^{Kip1}$ to be sequestered from the nucleus by cytoplasmic cyclinE-Cdk2 complexes. The overexpression of cyclin D retained $p27^{Kip1}$ in the cytoplasm (12, 25). From these observations, $p27^{Kip1}$ and cyclin-Cdk complexes seem to sequester each other in the cytoplasm. The NLS function is also known to be regulated by the phosphorylation of S or T close to NLS. For example, the phosphorylation of a casein kinase II site near the NLS of SV40 T-antigen increased the rate of nuclear translocation (26), while the nuclear transport of lamin B2 was inhibited by phosphorylation at the protein kinase C sites (27). In the sequence of $p27^{Kip1}$, several possible phosphorylation sites (T149, T157, T179, S160, S161) were found near the NLS. However, the involvement of phosphorylation in the regulation of subcellular localization of $p27^{Kip1}$ remains to be elucidated.

In conclusion, we herein clarified that a specific region, 153–166, to be the minimal sequence necessary to achieve nuclear localization, and this region is considered to be consistent with a consensus sequence of bipartite NLS. Four basic residues in this region, especially R166, play a critical role. However, the minimal region alone was found to be insufficient to achieve the full function of NLS, and thus suggesting some extension to be auxiliary. The precise mechanism underlying the regulation of the subcellular localization of $p27^{Kip1}$, however, remains to be clarified.

ACKNOWLEDGMENTS

We thank Mr. Brian Quinn for comments and help with the manuscript. This study was supported in part by Grants-in-Aid for Scientific Research (Nos. 10557072, 11838013, and 11670687) and for Scientific Research on Priority Area (No. 12213103) from the Ministry of Education, Science, Sports, and Culture, Japan, by the Research Grant for Cardiovascular Diseases (11C-1) from the Ministry of Health and Welfare, Japan, and by grants from the Foundation for the Promotion of Clinical Medicine, the Suzuken Memorial Foundation, and KANZAWA Medical Research Foundation.

REFERENCES

1. Sherr, C. J. (1994) *Cell* **79,** 551–555.
2. Pardee, A. B. (1989) *Science* **246,** 603–608.
3. Grana, X., and Reddy, E. P. (1995) *Oncogene* **11,** 211–219.
4. Pines, J. (1995) *Biochem. J* **308,** 697–711.
5. Sherr, C. J., and Roberts, J. M. (1995) *Genes Dev.* **9,** 1149–1163.
6. Hall, M., Bates, S., and Peters, G. (1995) *Oncogene* **11,** 1581–1588.
7. Polyak, K., Kato, J. Y., Solomon, M. J., Sherr, C. J., Massague, J., Roberts, J. M., and Koff, A. (1994) *Genes Dev.* **8,** 9–22.
8. Coats, S., Flanagan, W. M., Nourse, J., and Roberts, J. M. (1996) *Science* **272,** 877–880.
9. Singh, S. P., *et al.* (1998) *Cancer Res.* **58,** 1730–1735.
10. Orend, G., Hunter, T., and Ruoslahti, E. (1998) *Oncogene* **16,** 2575–2583.
11. Wang, G., Miskimins, R., and Miskimins, W. K. (1999) *Oncogene* **18,** 5204–5210.
12. Albrecht, J. H., Rieland, B. M., Nelsen, C. J., and Ahonen, C. L. (1999) *Am. J. Physiol.* **277,** G1207–G1216.
13. Loubat, A., Rochet, N., Turchi, L., Rezzonico, R., Far, D. F., Auberger, P., Rossi, B., and Ponzio, G. (1999) *Oncogene* **18,** 3324–3333.
14. Davis, L. I. (1995) *Ann. Rev. Biochem.* **64,** 865–896.
15. Nigg, E. A. (1997) *Nature* **386,** 779–787.
16. Dingwall, C., and Laskey, R. A. (1991) *Trends Biochem. Sci.* **16,** 478–481.
17. Kalderon, D., Roberts, B. L., Richardson, W. D., and Smith, A. E. (1984) *Cell* **39,** 499–509.
18. Gorlich, D., and Mattaj, I. W. (1996) *Science* **271,** 1513–1518.
19. Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massague, J. (1994) *Cell* **78,** 59–66.
20. Kawasaki, J., Hirano, K., Hirano, M., Nishimura, J., Nakatsuka, A., Fujishima, M., and Kanaide, H. (2000) *Eur. J. Pharmacol.*, in press.
21. Hirano, M., Niiro, N., Hirano, K., Nishimura, J., Hartshorne, D. J., and Kanaide, H. (1999) *Biochem. Biophys. Res. Commun.* **254,** 490–496.
22. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene* **77,** 51–59.
23. Conti, E., Uy, M., Leighton, L., Blobel, G., and Kuriyan, J. (1998) *Cell* **94,** 193–204.
24. Miyamoto, Y., Imamoto, N., Sekimoto, T., Tachibana, T., Seki, T., Tada, S., Enomoto, T., and Yoneda, Y. (1997) *J. Biol. Chem.* **272,** 26375–26381.
25. Baldassarre, G., et al. (1999) *J. Clin. Invest.* **104,** 865–874.
26. Jans, D. A. (1995) *Biochem. J* **311,** 705–716.
27. Hennekes, H., Peter, M., Weber, K., and Nigg, E. A. (1993) *J. Cell Biol.* **120,** 1293–1304.