Polo-like kinase 1 (Plk1), a mammalian ortholog of Drosophila Polo, is a serine-threonine protein kinase implicated in the regulation of multiple aspects of mitosis. The protein level, activity, and localization of Plk1 change during the cell cycle, and its proper subcellular localization is thought to be crucial for its function. Although localization of Plk1 to the centrosome has been established, nuclear localization or nucleocytoplasmic translocation of Plk1 has not been fully addressed. Here we show that Plk1 accumulates in both the nucleus and the cytoplasm in addition to its localization to the centrosome during S and G2 phases. Our results identify a conserved region in the kinase domain of Plk1 (residues 134–146) as a functional bipartite nuclear localization signal (NLS) sequence that regulates nuclear translocation of Plk1. The identified NLS is necessary and sufficient for directing nuclear localization of Plk1. This bipartite NLS has an unusually short spacer sequence between two clusters of basic amino acids but is sensitive to RanQ69L, a dominant negative form of Ran, similar to ordinary bipartite NLS. Remarkably, the expression of an NLS-disrupted mutant of Plk1 during S phase was found to arrest the cells in G1 phase. These results suggest that the bipartite NLS-dependent nuclear localization of Plk1 before mitosis is important for ensuring normal cell cycle progression.

In this study, we have demonstrated localization of Plk1 to the nucleus as well as to the cytoplasm during S and G2 phases and identified for the first time a bipartite nuclear localization signal sequence in Plk1 that regulates nuclear translocation of Plk1. In addition, we present several lines of evidence suggesting that nuclear localization of Plk1 before mitosis is important for ensuring normal cell cycle progression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Synchronization, and Transient Transfection**—HeLa cells were cultured and synchronized with double thymidine block as previously described (23). HeLa cells were transiently transfected by the use of FuGENE 6 according to the manufacturer's instructions with the use of 2 μg of total DNA/35-mm dish. After 20 h, cells were fixed and stained.

**Cell Fractionation**—Fractionation of HeLa cell extracts in each time after release from double thymidine block was performed as described previously (24). The nuclear and cytoplasmic fractions were analyzed by SDS-PAGE and immunoblotting. The nuclear and cytoplasmic fractions were normalized to contain equal quantity of total proteins.

**DNA Constructs**—The mutagenesis of Plk1 3A was performed using a mutagenic primer 5'-TTTGGAGCTGTTGCGCTCCTG-3' by the use of QuickChange site-directed mutagenesis kit (Stratagene). To yield Plk1 4A, a mutagenic primer 5'-CGGGATCCGACTTTGTATTTGTAG-3' was used. PCR products of Plk1 WT, 3A, 4A, and 7A were subcloned into pSR-HA vector. The mutations were confirmed by DNA sequencing. A sequence corresponding to residues 124–148 of human Plk1 was amplified by PCR with a 5' primer 5'-GGGATCCGACGGACTTTTAGTAGAG-3' and a 3' primer 5'-GGGATCCGACGGACTTTTAGTAGAG-3', generating BamHI and SalI sites at the 5' and 3' ends, respectively, and the fragment was

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The abbreviations used are: Plk1, Polo-like kinase 1; NLS, nuclear localization signal; GST, glutathione S-transferase; WT, wild type; HA, hemagglutinin; TRITC, tetramethylrhodamine isothiocyanate; BSA, bovine serum albumin; GFP, green fluorescent protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MPM2, mitotic phosphoprotein monoclonal-2. 

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inserted into pGEX-6P1 (Amersham Biosciences) to obtain GST-Plk1 (25 amino acids).

Recombinant Proteins—GST-Plk1 WT, GST-Plk1 7A, and GST-Plk1 (25 amino acids) were bacterially expressed and purified on glutathione-Sepharose 4B (Amersham Biosciences) as described previously (23).

Microinjection—GST-Plk1 proteins were injected into the cytoplasm of HeLa cells as described previously (25). A t 1 h after injection, cells were fixed and stained with anti-GST antibody (Santa Cruz Biotechnology). SR/H9251-HA-Plk1 WT or 7A was injected into the nucleus of HeLa cells at 4 h after the release from double thymidine block.

Cell Staining—Indirect immunofluorescent staining for endogenous Plk1 was performed as described previously (6). Cells at each time after release from double thymidine block were stained with rabbit anti-Plk1 antibody (Zymed Laboratories Inc.) and mouse anti-α/tubulin antibody (Clontech). Transfected or injected SRα-HA-Plk1 was stained with rabbit anti-HA antibody (Clontech) as described previously (23). Injected cells were also stained with mouse anti-cyclin B1 antibody (Santa Cruz Biotechnology), mouse anti-MPM2 antibody (UBI), and rabbit anti-phospho-(Tyr-15)cDC2 antibody in each experiment.

RESULTS AND DISCUSSION

Plk1 Enters the Nucleus during Interphase—To examine subcellular localization of endogenous Plk1 during interphase, we used the indirect immunofluorescent staining and the biochemical cell fractionation method in synchronized HeLa cells. Consistent with previous observations (6–11), the protein level of Plk1 is very low in G1/S phase, but its localization to the centrosome is detectable (Fig. 1, 0h). In S and G2 phases, Plk1 increases dramatically and localizes to both the cytoplasm and the nucleus in addition to the centrosome (Fig. 1, 4h and 6h). At prophase, the protein level of Plk1 is maximal and it shows both nuclear and cytoplasmic localization (Fig. 1, 8h). Thus, these observations indicate that Plk1 has the ability to enter the nucleus constitutively during interphase.

Identification of a Bipartite Nuclear Localization Signal in Plk1—It is generally believed that those proteins whose molecular mass is larger than 50 kDa cannot pass through the nuclear pore by passive diffusion. Because Plk1 is ~67 kDa, Plk1 should have some mechanism to enter the nucleus. We found a region in human Plk1 where two clusters of basic amino acids, residues 134–136 (RRR) and residues 143–146 (KRRK), are aligned in tandem (residues 143–146 (KRRK), are aligned in tandem (Fig. 2A). This region lies in the kinase catalytic domain in the primary sequence, and its se-
sequence is highly conserved in Xenopus Plx1 and Drosophila Polo (Fig. 2A). The yeast orthologs, Cdc5 and Plo1, lack the first cluster of basic amino acids but contain the second one (Fig. 2A). We hypothesized that this region in mammalian Plk1 might function as a bipartite NLS sequence.

To test this possibility, we first constructed a fusion protein between GST and a sequence corresponding to residues 124–148 of Plk1 (GST-Plk1 WT). After 20 h, cells were fixed and stained with rabbit anti-HA antibody (Clontech) and Hoechst 33342. Transfected cells with Plk1 WT, 3A, 4A, or 7A were scored with listed phenotypes. N>C, cells showing higher nuclear staining; N = C, cells showing nearly equal staining intensities in the nucleus and the cytoplasm; N<C, cells showing lower nuclear staining. The data were obtained from two independent experiments. Approximately 100 cells were scored in each condition.

To assess the importance of the first and second clusters of basic amino acids in the identified NLS sequence of Plk1, we made several mutant forms of Plk1 in which the first, second, and third clusters of basic amino acids were mutated to alanines. When injected into the cytoplasm, GST-Plk1 WT translocated to and accumulated in the nucleus, whereas GST-Plk1 7A did not translocate to the nucleus and remained in the cytoplasm. These results indicate that the identified NLS sequence is required for Plk1 to enter the nucleus.

FIG. 4. Expression of Plk1 WT, Plk1 3A, Plk1 4A, or Plk1 7A in HeLa Cells. A, HeLa cells were transiently transfected with SRα-HA-Plk1 WT, 3A, 4A, or 7A. After 20 h, cells were fixed and stained with rabbit anti-HA antibody (Clontech) and Hoechst 33342. B, transfected cells with Plk1 WT, 3A, 4A, or 7A were scored with listed phenotypes. N>C, cells showing higher nuclear staining; N = C, cells showing nearly equal staining intensities in the nucleus and the cytoplasm; N<C, cells showing lower nuclear staining. The data were obtained from two independent experiments. Approximately 100 cells were scored in each condition.

FIG. 5. Effect of expression of Plk1 7A during S phase. SRα-HA-Plk1 WT or 7A was injected into the nucleus of HeLa cells at 4 h after the release from double thymidine block. At 8 h after injection, cells were fixed and stained with anti-HA antibody and Hoechst 33342. A, typical images of HeLa cells expressing Plk1 WT or 7A. Arrowheads indicate some of injected cells. B, percentages of cells in mitosis in transfected cells with each plasmid. At least 70 cells were scored for each plasmid in each experiment. The data are obtained from three independent experiments.

FIG. 6. Expression of Plk1 7A arrests the cells in G2 phase. HeLa cells were treated as in Fig. 5. A, Plk1 7A-injected cells were stained with anti-HA antibody, Hoechst 33342, and anti-cyclin B1 antibody. B, HeLa cells were stained with anti-HA antibody, Hoechst 33342, and anti-MPM2 antibody. A right panel shows the percentages of MPM2-positive cells. C, HeLa cells were stained with anti-HA antibody, Hoechst 33342, and anti-phospho-(Tyr-15)cyclin B1 antibody. A right panel shows the percentages of phospho-(Tyr-15)cyclin B1-positive cells. In B and C, at least 70 cells were scored for each plasmid in each experiment. The data are obtained from three independent experiments.

To assess the importance of the first and second clusters of basic amino acids in the identified NLS sequence of Plk1, we made several mutant forms of Plk1 in which the first, second,
or both basic amino acid cluster(s) was mutated to alanines, respectively (see Fig. 2B). We then expressed HA-tagged forms of these mutants (3A, 4A, or 7A) and wild-type Plk1 in HeLa cells and examined their subcellular distribution by staining with anti-HA antibody. In more than half of the transfected cells, Plk1 WT accumulated in the nucleus, and in the remainder, Plk1 WT showed pan-cellular distribution (Fig. 4, WT). The mutation at the first basic cluster slightly affected the subcellular distribution. In ~40% of the transfected cells, Plk1 3A showed nuclear accumulation (Fig. 4, 3A). In contrast, nuclear accumulation was markedly impaired in the mutation at the second basic cluster. Plk1 4A showed pan-cellular distribution in ~80% of transfected cells, and in ~20% of the cells, it showed exclusively cytoplasmic localization (Fig. 4, 4A). The double mutation abolished completely nuclear localization. Plk1 7A showed exclusively cytoplasmic localization (Fig. 4, 7A). These results indicate that both the first and the second basic clusters contribute to nuclear translocation of Plk1, and therefore the identified NLS is in fact a bipartite NLS. In addition, the obtained data suggest that the second basic cluster alone can work as NLS although not so strong. It should be noted that the second basic cluster is conserved even in yeast orthologs of Plk1 (see Fig. 2A).

Expression of Plk1 7A Arrests the Cells in G2 Phase—To know possible physiological significance of nuclear localization of Plk1 during interphase, we examined the affect of expression of the NLS-disrupted mutant of Plk1 (Plk1 7A) on the cell cycle progression. We injected a plasmid harboring Plk1 7A or Plk1 WT into the nucleus of HeLa cells at 4 h after release from double thymidine block, and we followed the cell cycle progression by examining the state of chromosomes. A pGFP empty vector was injected as a control. Unlike un.injected HeLa cells entered the M phase at ~10 h after the release from the double thymidine block, the injection procedure under our conditions appeared to induce a slight delay (from 1 to 2 h) in the cell cycle progression. Approximately half of the empty vector-injected cells (~56%) was in M phase at 12 h (Fig. 5B, Vector). The expression of Plk1 WT did not affect the timing of M phase entry significantly. 48% of the cells expressing Plk1 WT were in M phase at 12 h (Fig. 5A and B, WT). In contrast, the expression of Plk1 7A seemed to block M phase entry. Only 5 to ~10% of the cells expressing Plk1 7A were in M phase at 12 h (Fig. 5A and B, 7A), and the majority of the cells had a large G2-like nucleus and grew to cover a large surface area (Fig. 5A, 7A). At 14 h, there was no increase in M phase cells in the cells expressing Plk1 7A, whereas most of the cells expressing Plk1 WT or the pGFP vector-injected cells went through mitosis and entered the G1 phase. Rather surprisingly, the cells expressing Plk1 7A did not enter the M phase and appeared to stay in G2 phase even at 24 h after release (data not shown).

To examine whether the cells expressing Plk1 7A were arrested in G2, we first checked subcellular localization of cyclin B1, which normally translocates from the cytoplasm to the nucleus before nuclear envelope breakdown during prophase (23, 26–29). At 12 h after the release, all of the Plk1 7A-injected cells (n = 65) showed exclusively cytoplasmic localization of cyclin B1 (Fig. 6A), suggesting that the cells are arrested in G2. We then performed cell staining with anti-MPM2 antibody, which is believed to recognize mitotic phosphoproteins. Although ~50% pGFP- or Plk1 WT-injected cells showed strong staining with this antibody at 12 h, few cells expressing Plk1 7A (<4%) were MPM2-positive (Fig. 6B). This result also suggests that the cells expressing Plk1 7A did not enter the M phase. Finally, we assessed the activation state of M-phase promoting factor by examining the phosphorylation state of Cdc2 on Tyr-15. The cell staining with anti-phospho-Tyr-15 Cdc2 antibody demonstrated that although >60% pGFP- or Plk1 WT-injected cells were negative for this staining, only ~20% Plk1–7A-injected cells were negative (Fig. 6C). This suggests that Cdc2 was not activated in most of the cells expressing Plk1 7A, consistent with the idea that the cells are arrested in G2.

We have here demonstrated that Plk1 localizes to both the cytoplasm and the nucleus in addition to the centrosome in S and G2 phases. Moreover, our results have identified a bipartite NLS that is responsible for nuclear localization of Plk1. Remarkably, the expression of an NLS-disrupted mutant of Plk1, Plk1 7A, during S phase is found to arrest the cells in G2 phase. These results suggest that nuclear localization of Plk1 directed by its NLS during interphase is required for cells to enter mitosis. In a recent study in which the injection of anti-Plk1 antibodies into cultured cells arrested the cells in G2, Lane and Nigg (30) suggested the existence of a centrosome-maturation checkpoint that is sensitive to the impairment of Plk1 function in cells (31). Similarly, we also hypothesize that there may be some checkpoint mechanism that senses nuclear localization of Plk1 or its interacting protein(s). Because the expression of Plk1 7A does not affect subcellular localization of endogenous Plk1,2 it is possible that expression of Plk1 7A, which is exclusively cytoplasmic, may retain some interacting protein(s) in the cytoplasm by competing with endogenous Plk1 and thereby could trigger activation of an assumed checkpoint pathway, resulting in G2 arrest. To examine whether localizing Plk1 7A in the nucleus rescues the G2 arrest, we added an NLS sequence of SV40 large T-antigen (PKKKRKVEDP) to the C terminus of the Plk1 7A (Plk1 7A-SV40NLS) and injected the plasmid harboring Plk1 7A-SV40NLS into the nuclei of HeLa cells. Unfortunately, however, this mutant Plk1 did not accumulate strongly in the nucleus but showed pan-cellular distribution. In addition, the expression of this mutant also induced the G2 arrest.2 Therefore, this experiment could not determine propriety of our simple hypothesis. On the other hand, more complicated scenarios are also possible. We cannot completely exclude the possibility that Plk1 7A might not be activated properly during the G2 to M phase transition, although Plk1 7A has essentially the same basal kinase activity as Plk1 WT.3 The NLS of Plk1 described here is located within the subdomain V of the kinase catalytic domain. Only the sequence–in Plk1 but not the sequence in the subdomain V of other protein kinases, matches the consensus sequence of NLS sequences. According to the predicted three-dimensional structure of the catalytic domain of Plk1 by the Swiss model, the NLS identified here forms a helix at the surface of the activation loop. Thus, the NLS may be well positioned for interactions with the nuclear import transporters.

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