Partial purification and characterization of a protein kinase that is activated by nuclear localization signal peptides

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Received 20 November 1995; revised version received 27 December 1995

Abstract A nuclear localization signal (NLS) is required for the transport of karyophilic proteins from the cytoplasm to the nucleus. In this study, NLS was examined in terms of its effect on diverse cellular functions such as protein phosphorylation reactions. When synthetic peptides containing the NLS of SV40 T-antigen were injected into the cytoplasm of Xenopus oocytes, and the oocytes incubated with [^{32}P]phosphorus-containing medium, a 32 kDa protein was found to be preferentially phosphorylated in an NLS-dependent manner. The incubation of fractionated cytosolic extracts prepared from mouse Ehrlich ascites tumor cells with [γ-^{32}P]ATP in the presence of the NLS peptides, results in the stimulation of the phosphorylation of several proteins. Similar in vitro stimulation was observed by other functional NLS peptides such as those of polyoma virus T-antigen and nucleoplasmin. Little or no stimulation, however, was detected for peptides of mutant type and reverse type NLS of SV40 T-antigen, and the C-terminal portion of lamin B. Using an in vitro assay, the phosphorylation activity was fractionated chromatographically and a fraction was obtained which contained a high level of activity. The fraction was found to contain three major proteins having molecular masses of 95, 70, and 43 kDa. The in vivo and in vitro results are consistent with the existence of a protein kinase, called NLS kinase, that is specifically activated by NLS peptides.

Key words: Nuclear localization signal; Protein phosphorylation; Protein kinase

1. Introduction

Mediated nuclear protein import is a two step process comprising: (a) an ATP-independent binding to the cytoplasmic face of nuclear pores, and, (b) ATP-dependent translocation through nuclear pore complexes (NPCs) [1,2]. The selective nuclear transport of karyophilic proteins is directed by short amino acid sequences termed nuclear localization signals (NLSs) [3–8].

Recent reports [9–12] suggest that several cytosolic factors are required for nuclear protein import. In higher eukaryotes, a 70 kDa heat-shock cognate protein (hsc70) represents one such cytoplasmic factor, which has been demonstrated in both living cells [13] and in vitro transport systems [14–16].

Using a semi-intact cell assay system, Imamoto et al. recently demonstrated that an SV40 T-antigen NLS-containing karyophile forms a stable complex (nuclear pore-targeting complex: PTAC) with cytoplasmic components to target nuclear pores [17]. They also reported that a 58 kDa component of PTAC (PTAC 58), the mouse homologue of Xenopus importin 60 which is essential for the first step of nuclear protein import [18], has NLS-binding activity and that anti-PTAC 58 antibodies inhibited in vivo nuclear import [19]. The PTAC 58 was also found to be homologous to a number of proteins. Among these are the 54/56 kDa NLS-receptor from the cytosol of bovine erythrocytes, previously reported by Adam and Gerace [20], karyopherin α isolated from a Xenopus oocyte cytosolic subfraction by Radu et al. [21], and hSRP1 obtained by Weis et al. [22]. A 97 kDa component of PTAC (PTAC 97) was required for the docking of import substrates to nuclear rims in conjunction with PTAC 58 [23], and was found to be the mouse homologue of karyopherin β [24], importin 90 [25], and p97 [26].

Moore and Blobel, using an in vitro, semi-intact cell system, purified a 25 kDa protein as a translocation factor from fraction B of Xenopus oocyte cytosol and showed it to be a small GTP binding protein, Ran/TC4 [27]. RCC1 acts as a guanine nucleotide exchange factor for Ran/TC4 and tsBN2 is a temperature-sensitive RCC1 mutant cell line derived from the BHK cell line. It has a point mutation in RCC1 and arrests in G_1 or induces premature chromosome condensation at non-permissive temperatures [28,29]. Tachibana et al. reported a decrease in the efficiency of nuclear protein transport for tsBN2 cells cultured at non-permissive temperatures and the accumulation of a factor that suppresses nuclear import [30]. In this report, we present data which shows that the RCC1-Ran/TC4 system plays an important role in nuclear protein import in living mammalian cells.

Nuclear protein import would be expected to occur as the result of a co-operative effect of these molecules. It is unclear, however, whether this co-operation is regulated by cellular conditions. For example, the efficiency of nuclear protein transport may change during the cell cycle or in response to the amount of nuclear proteins to be transported. It is well known that protein phosphorylation constitutes an important mechanism for the rapid and efficient regulation of cellular functions and the structural or functional changes of proteins related to, for example, signal transduction in the cell and the cell cycle.

In this report, we examined the role of NLS in cellular protein phosphorylation. Protein phosphorylation was stimulated by NLS peptides in vivo and in vitro. We also report the identification and purification of a protein kinase which is activated by NLS peptides in higher eukaryotes, which we designate a nuclear localization signal-activated kinase (NLS kinase).

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SSDI 0014-5793(96)00010-5

2. Materials and methods

2.1. Synthetic peptides

Synthetic peptides of SV40 T-antigen wild type NLS (CYGGPKKKRKVEDP), its mutant type NLS (CYGGPKTKRKVEDP), its reverse type NLS (CYGGPDEVKRKKKP), nucleoplasmin NLS (AVKRPAATKKGQAKKKKLDC), polyoma virus T-antigen NLS (CDPPRTPVSRKRPRPAC), and the C-terminal portion of lamin B (AKEFHYPQQKSGNKNC) were purchased from the Peptide Institute (Osaka, Japan).

2.2. Microinjection into Xenopus oocytes and in vivo phosphorylation assay

50 nl of 20 mM synthetic peptides containing SV40 T-antigen wild type NLS or its transport incompetent mutant type NLS, dissolved in 50 mM HEPES (pH 7.3), were microinjected into the cytoplasm of unfertilized Xenopus oocytes (stageV–VI) in MBS (modified Barth solution: 88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 20 mM HEPES (pH 7.3)) using a micromanipulator (Narishige 1M6, MN-153). As a control, 50 nl of 50 mM HEPES (pH 7.3) was microinjected. Immediately after microinjection, the oocytes were labeled by treatment with 0.5 mCi/ml of [^{32}P]phosphorus in MBS for 30 min. These procedures were performed at 21°C. Oocytes were homogenized in two volumes of homogenization buffer (20 mM sodium pyrophosphate, 10 mM ethylene glycol-bis-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 mM $MgCl_2$, 100 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol (DTT), 10 μg/ml leupeptin, 1 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 20 mM Tris-HCl (pH 7.5)) and extracts were obtained by centrifugation initially at 5,000 × *g* for 5 min and finally at 300,000 × *g* for 30 min (model TLA100.2; Beckman Instruments) [31]. The resulting extracts were applied to 1/3 volume of DEAE-Sephacel equilibrated with homogenization buffer at 4°C [32] and eluted stepwise with a homogenization buffer containing 0.23 M, 1 M, and 2 M NaCl using a batch method. Each fraction was subjected to SDS-PAGE followed by autoradiography.

2.3. Kinase purification

Ehrlich ascites tumor cells, freshly harvested from the abdominal cavity of mice, were washed with cold 10 mM phosphate buffer (pH 7.4) containing 138 mM NaCl and 2.7 mM KCl and lysed in hypotonic buffer A (20 mM HEPES (pH 7.2), 10 mM KCl, 5 mM $MgCl_2$, 0.2 mM PMSF, 10 μg/ml leupeptin, and 2 μg/ml cytochalasin B) by 10 strokes of a homogenizer. The lysate was clarified by centrifugation at 200,000 × *g* for 90 min (model 50.2Ti; Beckman Instruments) and applied to DEAE-Sephacel equilibrated with buffer B (the same as buffer A but 30 mM in KCl). Materials were eluted stepwise with buffer B containing 130 mM, 230 mM, 530 mM, and 1 M KCl. The 530 mM KCl fraction was then applied to hydroxylapatite gel (HTP: Bio-Rad Laboratories) equilibrated with buffer B. This column was washed with buffer C [30 mM potassium phosphate (pH 7.2), 300 mM KCl, 5 mM $MgCl_2$, 0.2 mM PMSF, 10 μg/ml leupeptin], and the bound materials were then eluted with buffer C containing 75 mM, 125 mM, and 250 mM potassium phosphate, respectively. The 125 mM potassium phosphate fraction was applied to Sephacryl S-300 (Pharmacia Biotechnology Inc.) equilibrated with buffer B. Kinase fractions and fractions containing, prominently, a phosphorylated 34 kDa protein (p34) were separated on this column. The kinase fractions eluted as two peaks. Fractions containing p34 were collected as a substrate fraction. The first peak, which contained kinase with a higher activity, was applied to Mono-Q HR 5/5 column (Pharmacia Biotechnology Inc.) equilibrated with buffer D (20 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 300 mM KCl, 0.2 mM PMSF, 10 μg/ml leupeptin) and eluted using a linear gradient of 0.3–2 M KCl in buffer D by HPLC (Pharmacia LKB Biotechnology Inc.).

2.4. In vitro phosphorylation assay

In vitro phosphorylation reactions were performed with or without 0.5–1.0 mM peptides in the presence of 10 μM ATP containing 1 μCi of [γ-^{32}P]ATP in an assay buffer (50 mM Tris-HCl (pH 7.5), 30 mM KCl, 5 mM $MgCl_2$) at 30°C for 10 min. The reaction was terminated by the addition of Laemmli's sample buffer and then heating to boiling. After SDS-PAGE, bands were detected by autoradiography. Histone H1, protamine, myelin basic protein, caseine, and phosvitin (Sigma Chem.) were used as kinase substrates at a concentration of 0.5 mg/ml. Crude nuclear pore complex proteins, isolated from rat liver [33], were also examined as substrates at a concentration of 0.5 mg/ml using the same assay condition. Apparent protein aggregation was not observed under these conditions.

3. Results and discussion

Initial experiments were directed at determining if NLS itself affects intracellular protein phosphorylation in vivo. Immediately after the injection of 50 nl of a 20 mM peptide solution of SV40 T-antigen wild type NLS, its mutant type NLS, or buffer alone were injected into the cytoplasm of Xenopus oocytes, and the oocytes were then incubated in a medium containing [^{32}P]phoshorus for 30 min. The resulting extracts were first applied to DEAE-Sephacel and fractionated by stepwise elution with 0.23 M, 1 M, and 2 M NaCl. Each fraction was separated by SDS-PAGE followed by autoradiography. As

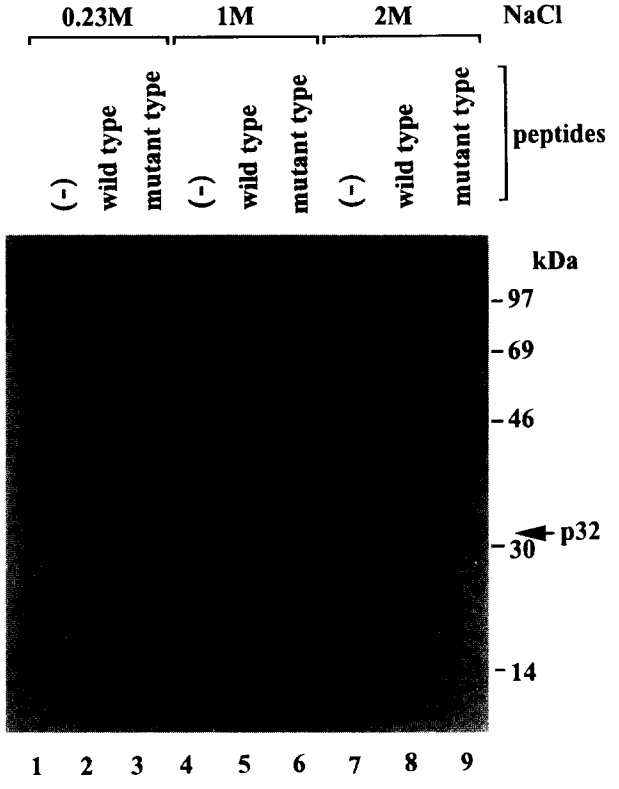

Fig. 1. Protein phosphorylation pattern in Xenopus oocytes injected with wild type NLS peptides of SV40 T-antigen. 50 nl of 20 mM synthetic peptides containing SV40 T-antigen wild type NLS (lanes 2, 5, and 8) or its mutant type NLS (lanes 3, 6, and 9) dissolved in 50 mM HEPES (pH 7.3) were microinjected into the cytoplasm of Xenopus oocytes (stage V–VI) in MBS by a micromanipulator. As a control, 50 nl of 50 mM HEPES (pH 7.3) (lanes 1, 4, and 7) was microinjected. Immediately after the microinjection, oocytes were labeled with 0.5 mCi/ml of [^{32}P]phosphorus in MBS for 30 min at 21°C. Labeled oocytes were homogenized in two volumes of 20 mM sodium pyrophosphate, 10 mM EGTA, 10 mM $MgCl_2$, 100 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 0.1 mM PMSF, and 20 mM Tris-HCl (pH 7.5) and the extracts were obtained by centrifugation first at 5,000 × *g* for 5 min and then at 300,000 × *g* for 30 min. These extracts were applied to 1/3 volume of DEAE-Sephacel at 4°C and eluted with buffer containing 0.23 M (lanes 1–3), 1 M (lanes 4–6), and 2 M (lanes 7–9) NaCl by batch method. Each fraction was subjected to SDS-PAGE followed by autoradiography. The arrow indicates the position of p32.

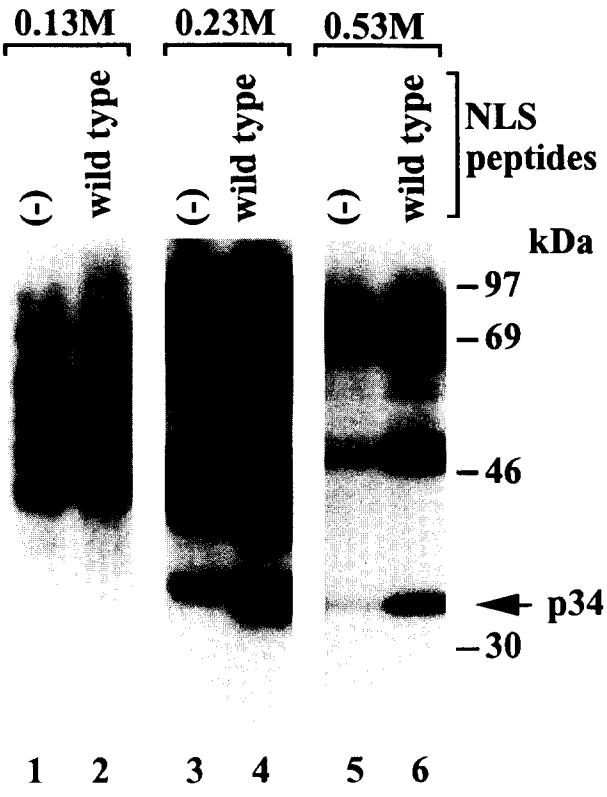

Fig. 2. In vitro phosphorylation with wild type NLS peptides of SV40 T-antigen. Ehrlich ascites tumor cells, freshly harvested from abdominal cavity of mice, were washed with 10 mM phosphate buffered saline and lysed in hypotonic buffer A (20 mM HEPES (pH 7.2), 10 mM KCl, 5 mM $MgCl_2$, 0.2 mM PMSF, 10 μg/ml leupeptin, and 2 μg/ml cytochalasin B) by 10 strokes of a homogenizer. The lysate was clarified by centrifugation at 200,000 × g for 90 min and applied to DEAE-Sephacel equilibrated with buffer B (the same as buffer A but 30 mM in KCl). Materials were eluted stepwise with buffer B containing 130 mM (lanes 1 and 2), 230 mM (lanes 3 and 4), and 530 mM (lanes 5 and 6) KCl. Each fraction was incubated with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) 1.0 mM wild type NLS peptides of SV40 T-antigen in the presence of 10 μM ATP containing 1 μCi of [γ-^{32}P]ATP in an assay buffer (50 mM Tris-HCl (pH 7.5), 30 mM KCl, 5 mM $MgCl_2$) at 30°C for 10 min. The reaction was terminated by the addition of Laemmli's sample buffer and boiling. After SDS-PAGE, bands were detected by autoradiography. The arrow indicates the position of p34.

shown in Fig. 1, several phosphorylated proteins were detected. Of these phosphorylated proteins, a 32 kDa protein (p32) was specifically and strongly phosphorylated as a result of the injection of wild type NLS peptides. For fractions obtained from oocytes injected with mutant type NLS peptides, minimal phosphorylation was observed. These results suggest the presence of a protein kinase which is activated in response to NLS peptides.

In order to verify this, we examined whether kinase activity could also be detected in vitro. Initially, we fractionated the cytosol of Ehrlich ascites tumor cells using DEAE Sephacel column chromatography (as described in section 2) and incubated each fraction with or without peptides in the presence of 10 μM ATP containing 1 μCi [γ-^{32}P]ATP in the assay buffer. As shown in Fig. 2, several proteins were specifically phosphorylated by the addition of peptides of SV40 T-antigen wild type NLS. Among these proteins, a 34 kDa protein (p34), from the 530 mM KCl fraction, was the major phosphorylated protein. As a result of this, the phosphorylation of p34 was used as an indicator of phosphorylation activity in subsequent experiments. In the next experiment, the 530 mM KCl fraction was applied to a hydroxyl apatite column and the bound materials were eluted using a stepwise gradient of potassium phosphate as described in section 2. The most abundant kinase activity was obtained in the fraction which was eluted with 125 mM potassium phosphate.

For further purification, the 125 mM fraction was subjected to gel filtration and in vitro phosphorylation was performed in each fraction. Phosphorylation of p34 was not detected in any of the fractions. Fractions which contained p34 were, however, obtained by visualization of the silver staining pattern of SDS-PAGE of each fraction. For confirmation, the p34-containing fractions were added to the other fractions and the in vitro phosphorylation reaction was performed in each mixture. As shown in Fig. 3, phosphorylation of p34 was detected in the mixtures and the kinase activity was separated into two peaks (peak I: about 360 kDa, peak II: about 150 kDa). These p34 containing fractions were also used as a substrate in further experiments. At the present time, it is not known whether p34 is a mouse homologue of p32 which is phosphorylated in Xenopus oocytes in an NLS-dependent manner as described above. Peak I was further separated by HPLC using a Mono-Q col-

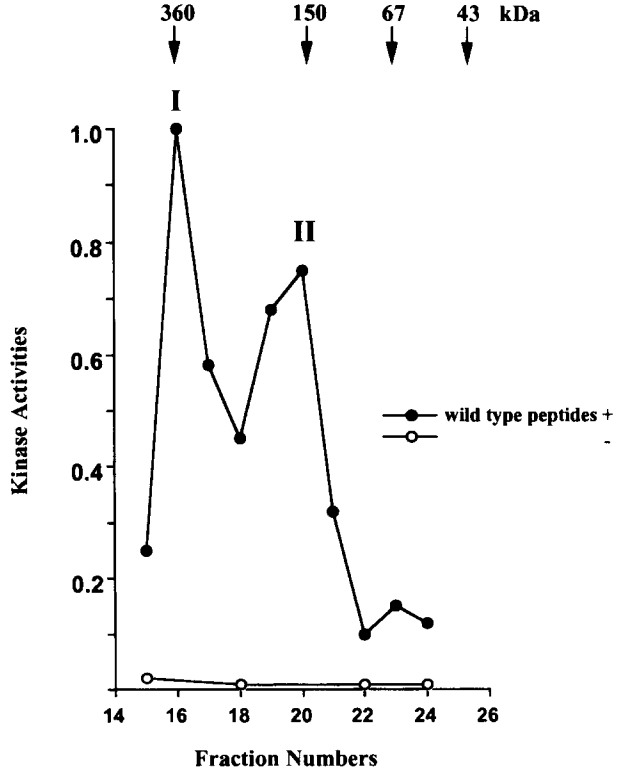

Fig. 3. Sephacryl S-300 chromatography of NLS kinase. The 125 mM fraction of hydroxylapatite column chromatography was applied to Sephacryl S-300 (Pharmacia Biotechnology Inc.) equilibrated with buffer B. Fractions containing p34 (p34 fractions) were detected by silver staining of SDS-PAGE of each fraction. The phosphorylation of p34 was not detected in each fraction. Each fraction was mixed with p34 fractions and an in vitro phosphorylation was carried out as described in section 2. Two peaks for the kinase activity were obtained. The positions of kinase activity are indicated as I and II. Kinase activity was quantified by measuring the intensity of bands of p34 with a scanning densitometer (CS-930, Shimazu, Japan) and the maximum activity of peak I was represented as 1.0.

umn with a sodium chloride gradient. Fractions containing kinase activity were detected in three major bands (95, 70, and 43 kDa) on SDS-PAGE by silver staining (Fig. 4A,B). These bands were not detected in fractions that were devoid of kinase activity.

The high molecular masses of the two kinase-active peaks obtained by gel filtration, and the fact that three molecular species having kinase activity were eluted from Mono-Q column, suggest that these molecules may form a complex which has kinase activity. Based on the present data, however, we cannot say with certainty whether the formation of complexes is necessary for kinase activity, or not. Furthermore, we cannot exclude the possibility that some minor proteins, detected by silver staining, might be responsible for the kinase activity.

The kinase fraction found in peak I was further characterized using the substrate fractions obtained by gel filtration. In order to confirm that the activation of the kinase is specific for NLS, we tested kinase activity by adding peptides of nucleoplasmin NLS and polyoma virus T-antigen NLS, which are competent for nuclear protein import, SV40 T-antigen transport incompetent mutant type NLS, reverse type NLS and the C-terminal

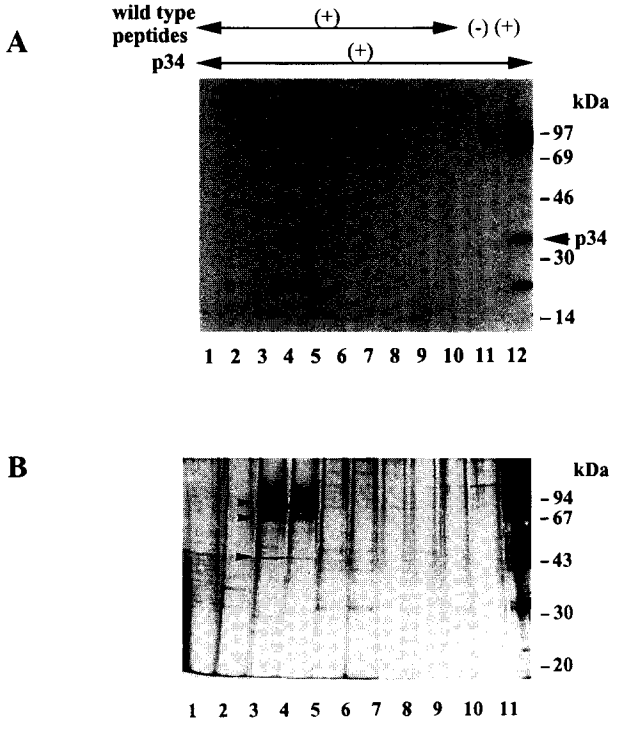

Fig. 4. Mono-Q high performance liquid chromatography of NLS kinase. The kinase peak I, which contained the most abundant activity, obtained by Sephacryl S-300 chromatography, was applied to Mono-Q HR 5/5 column (Pharmacia Biotechnology Inc.) equilibrated with buffer D (20 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 0.3 M KCl, 0.2 mM PMSF, 10 μg/ml leupeptin) and eluted with a linear gradient of 0.3–2 M KCl in buffer D by high performance liquid chromatography (HPLC)(Pharmacia LKB Biotechnology Inc.). (A) An in vitro phosphorylation assay was performed on samples (lanes 1–10) obtained after Mono-Q HPLC in the prescence of SV40 T-antigen wild type NLS peptides and p34 fractions obtained by Sephacryl S-300 gel filtration. Applied samples were mixed with p34 fractions and incubated with (lane 12) or without (lane 11) SV40 T-antigen wild type NLS peptides. The arrow indicates the position of p34. (B) The same samples as (A), lanes 1–11, were analysed by silver staining. Arrowheads indicate the position of major bands (95, 70, and 43 kDa) in the fractions which contain the kinase activity.

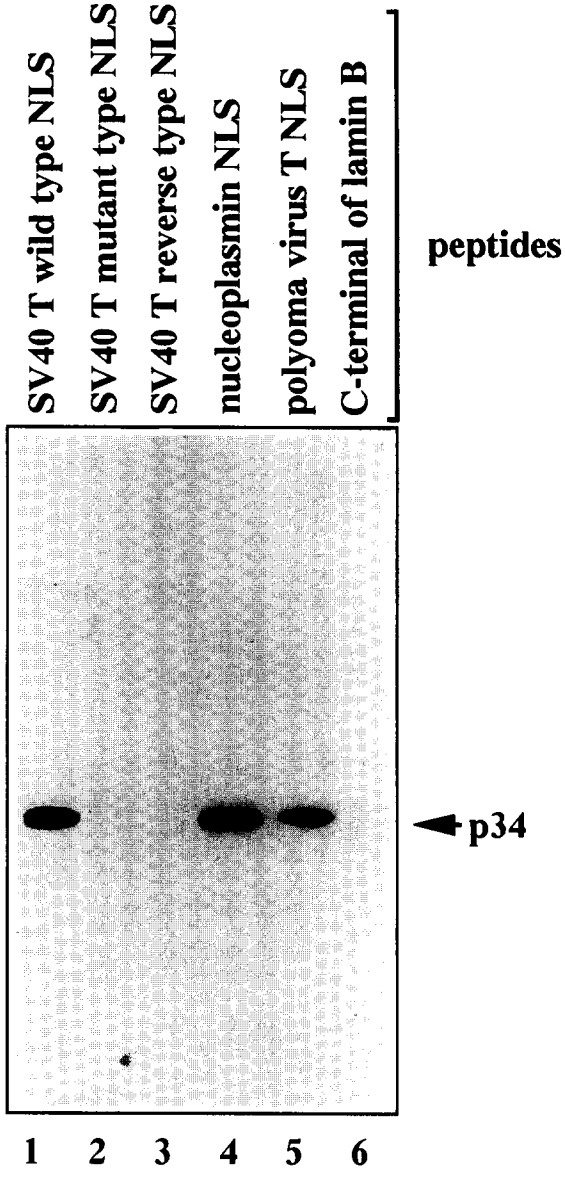

Fig. 5. NLS specificity of the kinase. Peptides of SV40 T-antigen wild type NLS (lane 1), mutant type NLS (lane 2), reverse type NLS (lane 3), nucleoplasmin NLS (lane 4), polyoma virus T-antigen NLS (lane 5), and the C-terminal portion of lamin B (lane 6) at the final concentration of 1.0 mM were added to the reaction mixtures containing peak I of the kinase and p34 substrate fractions obtained by gel filtration. An in vitro phosphorylation was performed as described in section 2. The arrow indicates the position of p34.

portion of lamin B, which contains no NLS region. As shown in Fig. 5, p34 was strongly phosphorylated by peptides of SV40 T-antigen wild type NLS, nucleoplasmin NLS, and polyoma virus T-antigen NLS, but not by peptides of SV40 T-antigen mutant type NLS, reverse type NLS, and the C-terminal portion of lamin B. These in vitro and in vivo results are fully consistent with the existence of a protein kinase which is activated by NLS peptides and which we refer to as nuclear localization signal-activated kinase (NLS kinase).

To further characterize the kinase, we examined whether common substrates of well-known kinases could be phosphorylated by the kinase. For these experiments, histone

H1, protamine, myelin basic protein, casein, and phosvitin were used as substrates for in vitro phosphorylation. None of these substrates were phosphorylated by the NLS kinase (data not shown). On the contrary, histone H1, a karyohile, stimulated kinase activity. This observation supports the hypothesis that the kinase can be activated by NLS, although the NLS of histone H1 has not yet been determined.

Very recently, Azuma et al. isolated a yeast protein kinase (Srp1p kinase) which is activated by Srp1p, a *Saccharomyces cerevisiae* homologue of importin 60, and showed the phosphorylation of Srp1p by Srp1p kinase was enhanced in the presence of NLS peptides [34]. Srp1p kinase phosphorylates a 36 kDa component of its protein kinase complex and its phosphorylation is not affected by NLS peptides. In contrast, no phosphorylation of any of the components of the NLS kinase fraction after Mono-Q column chromatography was observed even in the presence of NLS peptides. These data suggest that the NLS kinase is not a mouse homologue of Srp1p kinase. However, we have not yet examined whether PTAC 58 is phosphorylated by the NLS kinase.

Stochaj and Silver reported that the 70 kDa yeast NLS receptor lost NLS binding ability after treatment with phosphatase [35]. They showed that protein phosphorylation, which might produce either a direct modification of the NLS binding site or a change in protein conformation, was necessary for NLS-binding. Although ATP was not essential for the first step of the transport, the existence of the NLS kinase and its substrates in the cytosolic extracts suggest that this kinase might regulate the efficiency of nuclear pore targeting.

Mishra and Parnaik showed that treatment of semi-intact cells with alkaline phosphatase abolished nuclear protein import and that the insoluble structural proteins of 60–62 kDa of NPCs were predominantly phosphorylated by both protein kinase A and protein kinase C and that, after this phosphorylation, nuclear transport was restored [36]. Similarly, the NLS kinase might have an potential role in changing the conformation of NPCs by protein phosphorylation thus permitting karyophiles to bind to or translocate through the pores. In order to determine if NPCs can be phosphorylated by the NLS kinase, a crude fraction of NPCs, prepared from rat liver nuclei, was used as substrates in an in vitro assay. This crude fraction, however, was not phosphorylated under the conditions at which p34 was phosphorylated (data not shown). These findings, however, do not completely exclude the possibility that components of NPCs could serve as substrates for the NLS kinase, since this reaction was not studied by using more purified NPCs or over a wide range of assay conditions.

In terms of functionality, the NLS kinase may play an important role in the regulation of diverse cellular functions such as cell cycle control or signal transduction without a direct relationship to nuclear transport. Isolation of the genes for the NLS kinase and its substrate would, of course, lead to a more complete understanding of the exact cellular function and role of the kinase.

Acknowledgements: This work was supported by grants from the Japanese Ministry of Education, Science and Culture (07557057), and the Nissan Science Foundation.

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