Nuclear translocation of the Hsp70/Hsp90 organizing protein mSTI1 is regulated by cell cycle kinases

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Summary

The co-chaperone murine stress-inducible protein 1 (mSTI1), an Hsp70/Hsp90 organizing protein (Hop) homologue, mediates the assembly of the Hsp70/Hsp90 chaperone heterocomplex. The mSTI1 protein can be phosphorylated in vitro by cell cycle kinases proximal to a putative nuclear localization signal (NLS), which substantiated a predicted casein kinase II (CKII)-cdc2 kinase-NLS (CcN) motif at position 180-239 and suggested that mSTI1 might move between the cytoplasm and the nucleus under certain cell cycle conditions. The mechanism responsible for the cellular localization of mSTI1 was probed using NIH3T3 fibroblasts to investigate the localization of endogenous mSTI1 and enhanced green fluorescent protein (EGFP)-tagged mSTI1 mutants. Localization studies on cell lines stably expressing NLS^{mSTI1}-EGFP and EGFP demonstrated that the NLS^{mSTI1} was able to promote a nuclear localization of EGFP. The mSTI1 protein was exclusively cytoplasmic in most cells under normal conditions but was present in the nucleus of a subpopulation of cells and accumulated in the nucleus following inhibition of nuclear export (leptomycin B treatment). G1/S-phase arrest (using hydroxyurea) and inhibition of cdc2 kinase (using olomoucine) but not inhibition of casein kinase II (using 5,6-dichlorobenzimidazole riboside), increased the proportion of cells with endogenous mSTI1 nuclear staining. mSTI1-EGFP behaved identically to endogenous mSTI1. The functional importance of key residues was tested using modified mSTI1-EGFP proteins. Inactivation and phosphorylation mimicking of potential phosphorylation sites in mSTI1 altered the nuclear translocation. Mimicking of phosphorylation at the mSTI1 CKII phosphorylation site (S189E) promoted nuclear localization of mSTI1-EGFP. Mimicking phosphorylation at the cdc2 kinase phosphorylation site (T198E) promoted cytoplasmic localization of mSTI1-EGFP at the G1/S-phase transition,whereas removal of this site (T198A) promoted the nuclear localization of mSTI1-EGFP under the same conditions. These data provide the first evidence of nuclear import and export of a major Hsp70/Hsp90 co-chaperone and the regulation of this nuclear-cytoplasmic shuttling by cell cycle status and cell cycle kinases.

Key words: Sti1, Hop, Hsp70, Hsp90, Cdc2, CKII

Introduction

The co-chaperone murine stress-inducible protein 1 (mSTI1) is a homologue of the human heat-shock protein 70 (Hsp70) / heat-shock protein 90 (Hsp90) organizing protein (Hop) and yeast stress-inducible protein 1 (STI1) (Blatch et al., 1997). Hop acts as an adaptor protein, mediating the association of the molecular chaperones Hsp70 and Hsp90 via specific tetratricopeptide-repeat (TPR)-rich binding domains (Pratt and Toft, 1997; Scheufler et al., 2000). mSTI1 has been shown to associate with heat-shock cognate 70 (Hsc70) and Hsp90, and to localize mainly to the cytoplasm (Lässle et al., 1997; van der Spuy et al., 2000). However, recent reports have indicated that mSTI1 can also localize to the cell surface (Zanata et al., 2002) and to the lysosome surface (Agarraberes and Dice, 2001).

The mouse Hsp70 gene family has seven members. Cytoplasmic Hsc70, endoplasmic reticulum Grp78 (Bole et al., 1986) and mitochondrial Hsp70 (Michikawa et al., 1993) are constitutively expressed; Hsp70-1 (Dingwall et al., 1988) and Hsp70-3 (Perry et al., 1994) are heat inducible; and Hsp70-2 (Zakeri et al., 1990) and heat-shock cognate t (Hsc70t) (Hang and Fox, 1995) are developmentally regulated. Hsc70 assists the folding of client proteins including nascent polypeptides (Helmbrecht et al., 2000). It also facilitates protein transport across mitochondrial and nuclear membranes (Okuno et al., 1993) and is involved in ubiquitin-mediated degradation of proteins (Bercovich et al., 1997). The diverse functions of Hsc70 might include a role within the cell cycle, because Hsc70 expression is coupled by an unknown mechanism to cell-cycle position in exponentially growing HeLa cells (Hunt et al., 1999), with *Hsc70* mRNA beginning to accumulate in mid-G1-phase, peaking in late G1-phase and declining during S-phase. Similarly, mRNA levels are increased in serum-stimulated CV1 monkey cells (Sainis et al., 1994) and Rat1 cells (Sorger and Pelham, 1987), and protein studies have indicated that a translocation of Hsc70 into the nucleus occurs during S-phase (Milarski and Morimoto, 1986). Interestingly, expression of Hsc/Hsp70 has been shown to not be cell-cycle regulated (Whitfield et al., 2002).

Several chaperones have been shown to be phosphorylated by cell cycle kinases: murine Hsp25 and human Hsp27

are phosphorylated by MAPK-activated protein kinase 2 (MAPKAP kinase 2) (Stokoe et al., 1992), heat-shock transcription factor 1 (HSF1) by cell division cycle 2 (cdc2) kinase (Reindl et al., 1997) and Hsp90 (Lees-Miller and Anderson, 1989), calnexin (Ou et al., 1992) and the immunosuppressant FK506-binding protein 52 (FKBP52) (Miyata et al., 1997) by casein kinase II (CKII). The mSTI1 protein has been shown to be phosphorylated in vitro by cell cycle kinases, CKII and cdc2 kinase, at residues proximal to a putative nuclear localization signal (NLS), substantiating a predicted CKII-cdc2-NLS (CcN) motif (Longshaw et al., 2000). CKII is important in the transition of cells from the G0 phase to the G1 phase of the cell cycle, and for continuing passage through the early G1 phase (Pyerin, 1994). The mammalian cdc2 kinase, when complexed with cyclin B, shows high similarity to the invertebrate M-phase-promoting factor (MPF), which initiates mitosis (Matsumoto and Fujimoto, 1990). A range of proteins, including p53 (Bischoff et al., 1990), lamin (Loewinger and McKeon, 1988), nucleoplasmin (Robbins et al., 1991) and the early G1 transcription factor Swi5 (Jans et al., 1995), exhibit similar CcN motifs. The SV40 T-antigen CcN motif functions under dual regulation, with phosphorylation at the CKII site increasing the rate of NLS-dependent nuclear import (Jans and Jans, 1994) and phosphorylation at the cdc2 kinase site inhibiting transport and markedly reducing the level of maximal nuclear accumulation (Jans et al., 1991). The inhibition by cdc2 kinase phosphorylation is thought to be through cytoplasmic retention or NLS masking, whereas the CKII phosphorylation-mediated enhancement might act through an increased affinity of association with the karyopherin complex, enhancing the docking rate at the nuclear pore complex (NPC) (Jans and Jans, 1994).

There is evidence for the existence of in vivo phosphorylated forms of mSTI1 and its homologues, Hop and STI1. The isoform compositions of Hop and mSTI1 shifted to more acidic forms after viral transformation and heat stress, respectively (Honore et al., 1992; Lässle et al., 1997), suggesting the stress-induced phosphorylation of a Hop/mSTI1 population. In addition, the major acidic variants of Hop have been shown to be phosphorylated (Honore et al., 1992). An acidic isoform of yeast STI1 has also been demonstrated, suggesting post-translational phosphorylation of STI1 (Nicolet and Craig, 1989).

The in vitro phosphorylation of mSTI1 in the vicinity of the proposed NLS by cell cycle kinases suggested a cell-cycle-dependent regulation of the nuclear localization of mSTI1 (Longshaw et al., 2000). Furthermore, the cytoplasmic localization of mSTI1 (Lässle et al., 1997) highlighted the proposed CcN motif as a potentially important regulator of mSTI1 localization, with relevance for the function of the Hsp70-mSTI1-Hsp90 multichaperone machine. Here, we provide evidence for a functional NLS within mSTI1 and the regulation of mSTI1 nuclear localization by the cell cycle and cell cycle kinases.

Materials and Methods

General procedures

Common procedures such as polymerase chain reaction (PCR), ligation reactions, agarose gel electrophoresis, competent bacterial cell preparation, plasmid DNA isolation and restriction enzyme digestion were performed according to standard protocols (Sambrook and Russell, 2001). Sodium-dodecyl-sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) was according to Laemmli (Laemmli, 1970) and western blotting according to Towbin et al. (Towbin et al., 1979). Luminol-based chemiluminescence detection reagents (ECL; Amersham Pharmacia Biotech) were used for immunodetection.

Plasmid construction

The mammalian construct pB-mSTI1-EGFP encodes full-length mSTI1 as an N-terminal fusion with enhanced green fluorescent protein (EGFP). *mSTI1* cDNA was PCR amplified from template pGEX3X2000 (Lässle et al., 1997) (Expand High Fidelity PCR system; Roche) to introduce a Kozak sequence and remove the stop codon. The PCR product was subcloned into pGEM(T) Easy Vector System I (Promega), the *mSTI1* fragment subcloned into pCineoEGFP *Nhe*I/*Sac*II sites, the *mSTI1-EGFP* fragment subcloned into pSK *Nhe*I/*Not*I sites and finally into pBCMGSNeo (Karasuyama and Melchers, 1988) *Xho*I/*Not*I sites to produce pB-mSTI1-EGFP. pB-NLS^{mSTI1}-EGFP encodes the mSTI1 amino acid sequence 222-239 (which contains the potential bipartite NLS) as an N-terminal fusion with EGFP. *EGFP* cDNA was PCR amplified from the template pCineoEGFP with a primer encoding mSTI1 amino acids 222-239 and a Kozak sequence. The resulting PCR product was subcloned into pGEM(T) and the NLS^{mSTI1}-EGFP fragment cloned into the pBCMGSNeo *Xho*I/*Not*I sites to produce pB-NLS^{mSTI1}-EGFP. The pET5a2000 bacterial construct encodes full-length mSTI1 for heterologous expression in *Escherichia coli*. *mSTI1* cDNA was PCR amplified from the template pGEX3X2000, the PCR product subcloned into pGEM(T) and the *mSTI1* fragment cloned into pET5a (Stratagene) *Nde*I/*Nhe*I sites to produce pET5a2000.

Site-directed mutagenesis

pB-mSTI1(S189A)-EGFP and pB-mSTI1(T198A)-EGFP were generated to encode full-length mSTI1 as an N-terminal fusion with EGFP, with alanine substitutions at positions 189 and 198, respectively. Similarly pB-mSTI1(S189E)-EGFP and pB-mSTI1(T198E)-EGFP included glutamic acid substitutions at positions 189 and 198, respectively. Site-directed mutagenesis reactions were carried out using the template pSK-mSTI1-EGFP in a linear amplification reaction (Quikchange Site-directed Mutagenesis kit; Stratagene). Mutated *mSTI1-EGFP* cDNA fragments were cloned into pBCMGSNeo *Xho*I/*Not*I sites. Plasmid construct pET5a2000 was used as a template to produce mSTI1(T198A) and mSTI1(T198A,T332A) expressing derivative plasmids (pET5a2000T198A and pET5a2000T198A-T332A) in a similar linear amplification reaction.

Transfections

Mouse NIH3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, penicillin (100 U ml^{-1}) and streptomycin (100 U ml^{-1}) (complete media; Highveld Biological) in a humidified atmosphere, at 37°C with 10% CO_2. For episomally stable transfectants, NIH3T3 fibroblasts were seeded to 30-50% confluence, transfected by the calcium phosphate method and selected with geneticin (0.5 mg ml^{-1}) (Gibco-BRL). Transient transfections were carried out using Lipofectamine Plus (Gibco-BRL) according to the manufacturer's instructions. Cells were fixed in 3.7% formaldehyde for 10 minutes and nuclei stained with 2 μg ml^{-1} 4′, 6-diamidino-2-phenylindole (DAPI; Sigma) in PBS. Slides were mounted in fluorescent mounting solution (DAKO) and the fluorescence visualized with a Zeiss LSM 510 laser scanning confocal microscope. Quantitation of cells showing a particular localization pattern was determined for all the cell treatments by assessment of

approximately 100 cells in each of five separate fields by an observer who was blind to the experimental status.

Indirect immunofluorescence

24 hours after transient transfection, cells were fixed in 3.7% formaldehyde. After fixation, all incubation and wash buffers contained 0.1% saponin (Sigma) for permeabilization. To reduce non-specific cross-reactivity, cells were incubated in blocking buffer [3% bovine serum albumin (BSA), 10% normal donkey serum in PBS] for 30 minutes before incubation with rabbit anti-mSTI1 antibody SF1 (Lässle et al., 1997) 1:250 in blocking buffer. Secondary antibody incubations were performed with Cy3-conjugated donkey anti-rabbit antibody (Pierce) diluted 1:200 in blocking buffer, and nuclei were stained with DAPI. Slides were mounted in fluorescent mounting solution (DAKO) and the fluorescence visualized with a Zeiss LSM 510 laser scanning confocal microscope. The following excitation and emission conditions were used in separate channels with either a 40× or 63× oil-immersion objective: DAPI, 364 nm and 475-525 nm; FITC/GFP, 488 nm and 505-530 nm; Cy3, 543 nm and 560 nm. Cells showing a particular localization pattern were quantified for all the cell treatments by assessing approximately 100 cells in each of five separate fields by an observer who was blind to the experimental status.

Treatment of cells with kinase inhibitors and cell cycle inhibitors

NIH3T3 cells stably expressing mSTI1-EGFP, mSTI1(S189A)-EGFP, mSTI1(S189E)-EGFP, mSTI1(T198A)-EGFP and mSTI1(T198E)-EGFP were treated with cell-cycle inhibitors 24 hours after seeding. CKII was inhibited by incubating the cells with 0.3 mM 5,6-dichlorobenzimidazole riboside (DRB) and cdc2 kinase was inhibited by incubation with 0.3 mM olomoucine for 8 hours. Nuclear export was inhibited by the addition of 18.5 nM leptomycin B for 3 hours. Cells were arrested in G1/S phase by the addition of 10 mM hydroxyurea for 10 hours. No initial arrest in G0/G1 by the incubation of cells in serum-free media was performed because this would result in a stressed cellular state. Cells were fixed and proteins were visualized as before.

Immunoprecipitation of GFP-mSTI1 and its derivates

Cells were transfected with pB-mSTI1-EGFP, pB-mSTI1(S189A)-EGFP, pB-mSTI1(T198A)-EGFP, pB-mSTI1(S189E)-EGFP, pB-mSTI1(T198E)-EGFP or pB-EGFP. 24 hours after transfection, cells were washed twice in PBS and scraped into 500 μl modified RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 50 mM NaCl) containing a cocktail of protease inhibitors (Sigma). Following 30 minutes incubation at 4°C, cell lysates were centrifuged at 17,500 ***g*** for 15 minutes. The supernatant was incubated overnight at 4°C with the monoclonal anti-GFP antibody 3E6 (Molecular Probes) or with non-immune IgG. Before incubation, antibodies were immobilized to protein-G/Sepharose (Amersham Pharmacia Biotech) using the cross-linker DSS (Pierce) according to the manufacturers instructions. After the overnight incubation the protein-G/Sepharose was washed six times for 10 minutes each in modified RIPA buffer. After washing, bound proteins were eluted with ImmunoPure elution buffer (Pierce) and subjected to western-blot analyses. Immunoprecipitated mSTI1-EGFP and mSTI1 were detected with a polyclonal antibody raised against GST-mSTI1 at 1:1000 (Lässle et al., 1997). Co-immunoprecipitated Hsc70 and Hsp70 were detected with BRM-22 (Sigma) at 1:1000, and Hsp90 was detected with sc31119 at 1:500 (Santa Cruz Biotechnology).

Purification and phosphorylation of mSTI1 proteins

Soluble cell extract were prepared from induced cultures of *E. coli* BL21(DE3)[pET5a2000], *E. coli* BL21(DE3)[pET5a2000T198A] and *E. coli* BL21(DE3)[pET5a2000T198A-T332A] expressing mSTI1, mSTI1(T198A) and mSTI1(T198A,T332A), respectively. The cell extracts were loaded onto a 30 ml Polybuffer Exchanger PBE column (Amersham Pharmacia Biotech) pre-equilibrated in start buffer (20 mM Tris-HCl, pH 8.0). 1.5 ml fractions were collected at a flow rate of 30 ml hr^{-1}. The column was washed with start buffer (50 ml), wash buffer (50 ml; start buffer containing 0.1 M NaCl) and an elution gradient of 0.1-0.3 M NaCl in wash buffer (50 ml). All fractions were tested for the presence and purity of mSTI1 using western-blot analysis and SDS-PAGE. Cdc2 kinase assays were performed on purified mSTI1 according to Longshaw et al. (Longshaw et al., 2000), except that the assays were incubated for 3 hours and 9 hours.

Results

mSTI1 is predominantly cytoplasmic

We found, as shown previously (Lässle et al., 1997), that mSTI1 was predominantly localized to the cytoplasm (Fig. 1A, middle). To determine the subcellular localization of mSTI1 in a system that could be subsequently engineered to produce modified mSTI1 proteins, a fusion of mSTI1 with EGFP at its C-terminus (mSTI1-EGFP) was constructed. NIH3T3 mouse fibroblast cells stably expressing mSTI1-EGFP and EGFP were analysed by confocal laser fluorescence microscopy. mSTI1-EGFP was localized to the cytoplasm of most cells, whereas EGFP was found to localize to the nucleus and cytoplasm of all cells (Fig. 1B). In addition, exogenously expressed mSTI1-EGFP was found to co-localize with endogenous mSTI1 (Fig. 1A). This suggested that the fusion of EGFP to the C-terminus of mSTI1 had no significant effect on its subcellular distribution.

Amino acids 222-239 functioned as an NLS

Proteins larger than 40-60 kDa cannot enter the nucleus through the nuclear pore complex by passive diffusion (Okuno et al., 1993). Because mSTI1-EGFP (at 89 kDa) is too large and endogenous mSTI1 (at 63 kDa) is of borderline mass to diffuse into the nucleus, a functional nuclear localization signal (NLS) would be required for the active transport of mSTI1 into the nucleus. Yet we observed that endogenous mSTI1 and mSTI1-EGFP were present in the nucleus of 10-20% of the cells (e.g. Fig. 2B). To assess the potential of the proposed mSTI1 NLS to direct nuclear import, a construct encoding amino acids 222-239 fused to the N-terminus of EGFP was expressed in NIH3T3 cells. The expressed protein, denoted NLS^{mSTI1}-EGFP, localized strongly to the nucleus in all cells compared with the EGFP control, which was localized diffusely throughout the cytoplasm and nucleus in all cells (Fig. 1B). According to the theoretical size of NLS^{mSTI1}-EGFP (~30 kDa), one would expect that it could move freely between the nucleus and the cytoplasm by diffusion. Only a small proportion of the NLS^{mSTI1}-EGFP was observed in the cytoplasm, however, suggesting a constitutively active nuclear import process. These data therefore clearly suggested that amino acids 222-239 of mSTI1 contain a functional NLS.

Leptomycin B treatment induced nuclear accumulation of endogenous mSTI1

Although mSTI1 potentially has a functional NLS at positions

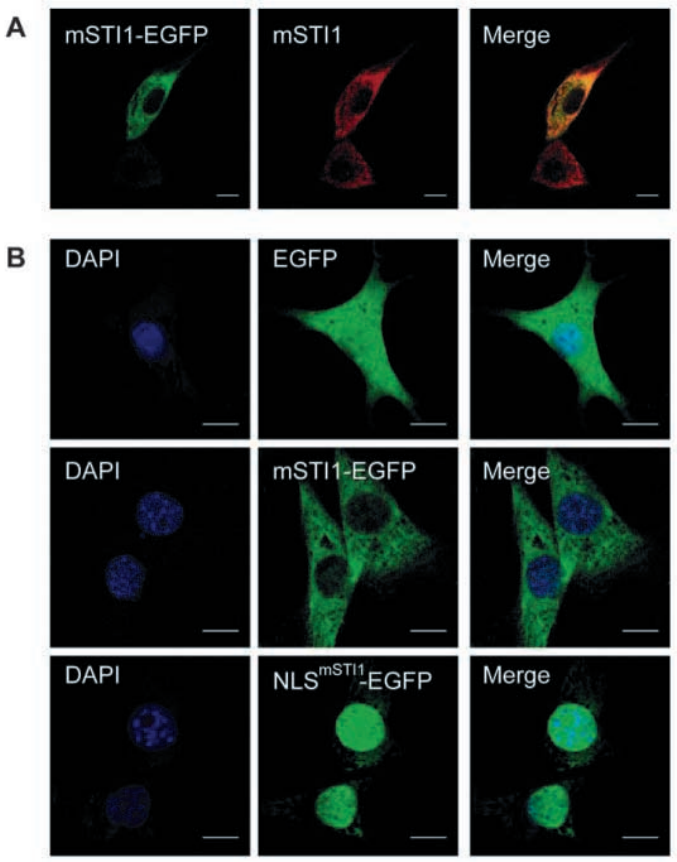


Fig. 1. The subcellular localization of mSTI1-EGFP and NLS^{mSTI1}-EGFP. (A) NIH3T3 fibroblasts were transiently transfected with pB-mSTI1-EGFP, fixed in 3.7% paraformaldehyde, permeabilized in 0.1% saponin (all panels) and immunostained with SF1 polyclonal rabbit anti-mSTI1 and Cy3-conjugated donkey anti-rabbit antibodies (middle and right panel). Cells were mounted and visualized by confocal laser fluorescence microscopy. Scale bars, 10 μm.
(B) NIH3T3 fibroblasts were stably transfected with pB-EGFP (top), pB-mSTI1-EGFP (middle) and pB-NLS^{mSTI1}-EGFP (bottom). Cells were fixed in 3.7% paraformaldehyde and stained with DAPI before visualization by confocal laser fluorescence microscopy. Scale bars, 10 μm.

222-239, it remains predominantly cytoplasmic under normal growth conditions (Figs 1, 2). This might be caused by the presence of a nuclear export system that predominates over nuclear import. To assess the potential nuclear export of mSTI1, NIH3T3 fibroblasts were treated with leptomycin B. Treatment with leptomycin B for 3 hours induced nuclear accumulation of endogenous mSTI1 in most cells (Fig. 2A,B). Similarly, in NIH3T3 stable transfectants expressing mSTI1-EGFP, treatment with leptomycin B induced nuclear accumulation of the fusion protein in most cells (Fig. 2A,B). These data demonstrated that mSTI1 was exported from the nucleus by a mechanism involving either a functional cis-acting, leptomycin-B-sensitive nuclear export signal (NES) or via a NES-containing interaction partner of mSTI1. The predominant cytoplasmic distribution of mSTI1 under normal conditions might therefore result from a dynamic equilibrium between nuclear import and export, in which the rate of export was higher in most cells.

Kinase inhibition affected the distribution of endogenous mSTI1

We have previously suggested that the CcN motif of mSTI1 might direct nuclear import under cell cycle control (Longshaw et al., 2000). The proposed CcN motif would comprise the NLS at position 222-239 together with the CKII (S189) and cdc2 kinase (T198) phosphorylation sites located upstream from the NLS and recognized in vitro. To assess any effect of specific inhibition of cdc2 kinase and CKII in cells on the cytoplasmic distribution of mSTI1, we exposed NIH3T3 fibroblasts to olomoucine (a specific inhibitor of cdc2 kinase) and DRB (a specific inhibitor of CKII). The incidence of cells with a cytoplasmic distribution of mSTI1 and mSTI1-EGFP was unchanged after DRB treatment, suggesting that inactivation of CKII did not change the distribution of mSTI1 (Fig. 2A,B). By contrast, olomoucine treatment resulted in a marked increase in the incidence of nuclear localization of mSTI1 (Fig. 2A,B). Similarly, treatment with olomoucine increased the incidence of nuclear localization of mSTI1-EGFP in NIH3T3 stable transfectants (Fig. 2A,B). These data suggested that active cdc2 kinase was required for the cytoplasmic accumulation of mSTI1.

Arrest at the G1/S-phase transition promoted a nuclear localization of endogenous mSTI1

We have previously identified a cdc2 kinase phosphorylation site in mSTI1 that was recognized in vitro (Longshaw et al., 2000) and, here, we have shown that the inhibition of cdc2 kinase led to an increase in the incidence of nuclear localization of mSTI1. We speculated that arrest of the cell at the G1/S-phase transition when cdc2 kinase was inactive might affect the localization of mSTI1. After hydroxyurea treatment, there was a marked increase in the incidence of nuclear localization of mSTI1 (Fig. 3A,B). Similarly, treatment with hydroxyurea increased the incidence of nuclear localization of exogenously expressed mSTI1-EGFP (Fig. 3A,B). These data again suggest that active cdc2 kinase was required for the cytoplasmic accumulation of mSTI1 and that the nuclear localization of mSTI1 was increased when the cells were in the G1/S-phase stage of the cell cycle.

Mimicking CKII S189 phosphorylation promoted nuclear localization of mSTI1-EGFP

Exogenously expressed mSTI1-EGFP showed the same localization characteristics as endogenous mSTI1, so we used modified derivatives of mSTI1-EGFP to investigate the mechanism of cytoplasmic-nuclear shuttling of mSTI1. To assess the effect of removal of the potential CKII phosphorylation site on mSTI1 localization, S189 was replaced with an alanine (S189A). The proportion of cells that demonstrated nuclear localization of expressed protein was found to be similar in cells expressing mSTI1(S189A)-EGFP (18%) and mSTI1-EGFP (13%) under normal conditions (Fig. 4A,B). Studies of the CcN motif in SV40 T-antigen have simulated phosphorylation by replacing the serine phosphorylation site with a negatively charged amino acid; this results in an enhancement of nuclear transport of the SV40 T-antigen (Jans and Jans, 1994).

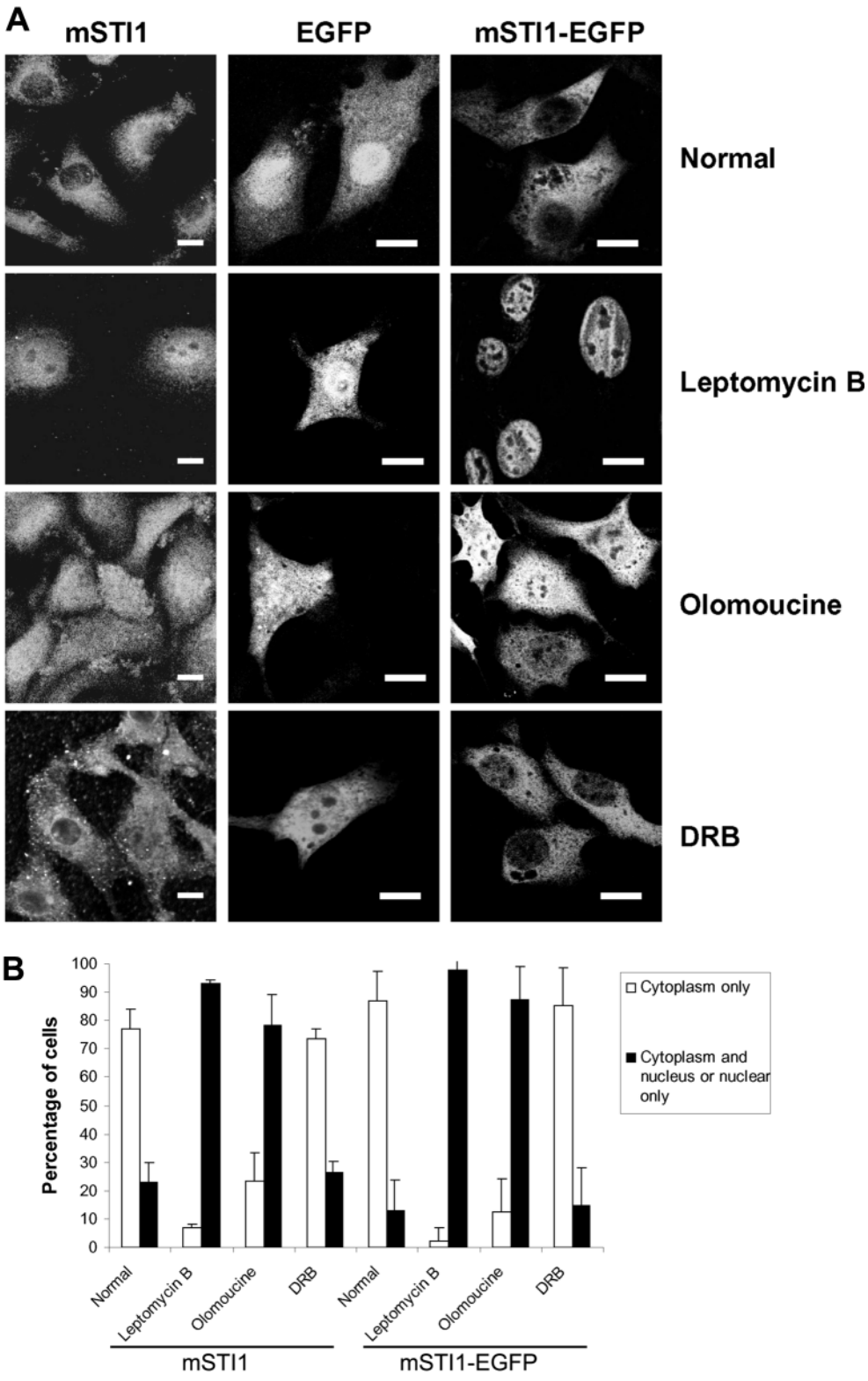
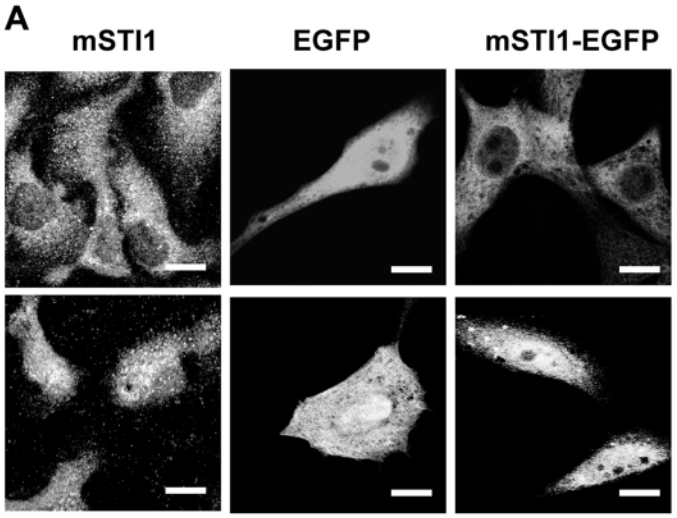


Fig. 2. The nuclear localization of mSTI1-EGFP is enhanced by inhibition of nuclear export and inhibition of cdc2 kinase but not by inhibition of CKII. (A) Untransfected NIH3T3 fibroblasts (left), NIH3T3 fibroblasts stably transfected with pB-EGFP (middle) or with pB-mSTI1-EGFP (right) were exposed to leptomycin B (18.5 nM), olomoucine (0.1 mM) or DRB (0.3 mM) for 3 hours, 8 hours and 8 hours, respectively. Untransfected cells were immunostained to detect endogenous mSTI1 with SF1 polyclonal rabbit anti-mSTI1 and Cy3-conjugated donkey anti-rabbit antibodies, and transfected cells were fixed in 3.7% paraformaldehyde before mounting and visualization by confocal laser fluorescence microscopy. Scale bars, 10 μm. (B) Cells as above were exposed to leptomycin B, olomoucine and DRB, and cells demonstrating cytoplasmic fluorescence or nuclear and cytoplasmic fluorescence were quantified. The error bars represent standard deviations.

Therefore, to assess the effect of phosphorylation at the CKII site on nuclear import of mSTI1-EGFP, the serine at position 189 was replaced with a glutamic acid (S189E). The proportion of cells that demonstrated increased nuclear localization of expressed protein was found to be greater in cells expressing mSTI1(S189E)-EGFP (44%) than in those expressing mSTI1-EGFP (13%) (Fig. 4A,B). This suggested an enhancing effect of a negative charge at the S189 site on mSTI1-EGFP nuclear import under normal conditions.



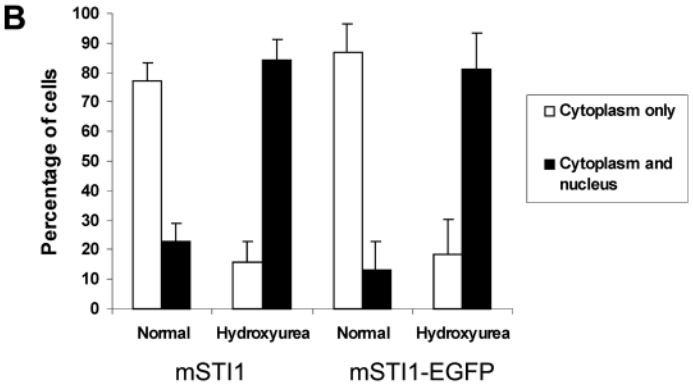


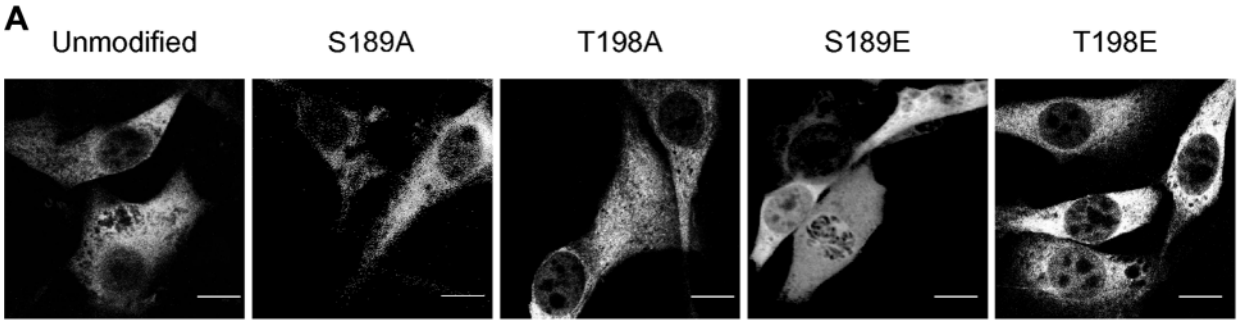
Fig. 3. The nuclear localization of endogenous mSTI1 and mSTI1-EGFP is enhanced by G1/S-phase arrest. (A) Untransfected NIH3T3 fibroblasts (left), NIH3T3 fibroblasts stably transfected with pB-EGFP (middle) or with pB-mSTI1-EGFP (right) were exposed to hydroxyurea (10 mM) for 10 hours. Untransfected cells were immunostained to detect endogenous mSTI1 with SF1 polyclonal rabbit anti-mSTI1 and Cy3-conjugated donkey anti-rabbit antibodies, and transfected cells were fixed in 3.7% paraformaldehyde before mounting and visualization by confocal laser fluorescence microscopy. Scale bars, 10 μm. (B) Cells were exposed to hydroxyurea as above, and cells demonstrating cytoplasmic fluorescence or nuclear and cytoplasmic fluorescence were quantified. The bars represent standard deviations.

Mimicking cdc2 kinase T198 phosphorylation promoted cytoplasmic localization of mSTI1-EGFP

To assess the effect of phosphorylation at the cdc2 kinase phosphorylation site on nuclear import of mSTI1, the threonine at position 198 was replaced with glutamic acid (T198E). Under normal growth conditions, mSTI1-EGFP and mSTI1(T198E)-EGFP were cytoplasmic in most cells (Fig. 4A,B). Following hydroxyurea treatment, however, the number of cells with nuclear localization of mSTI1-EGFP was found to be lower in cells expressing mSTI1(T198E)-EGFP (51%) than in those expressing mSTI1-EGFP (81%) (Fig. 5A,B). This suggested an inhibitory effect of a negative charge at the T198 site on mSTI1-EGFP nuclear import under G1/S-phase arrest. Alternatively, this negative charge might promote cytoplasmic retention of mSTI1-EGFP under G1/S-phase arrest.

Removal of the cdc2 kinase phosphorylation site promoted nuclear localization of mSTI1-EGFP

Specific inhibition of cdc2 kinase increased the nuclear localization of mSTI1, so we assessed the importance of the potential in vivo cdc2 kinase phosphorylation site T198 by replacing it with an alanine residue (T198A). The proportion of cells demonstrating cytoplasmic and nuclear localization of fluorescence was found to be similar in cells expressing mSTI1(T198A)-EGFP (11%) to cells expressing mSTI1-EGFP



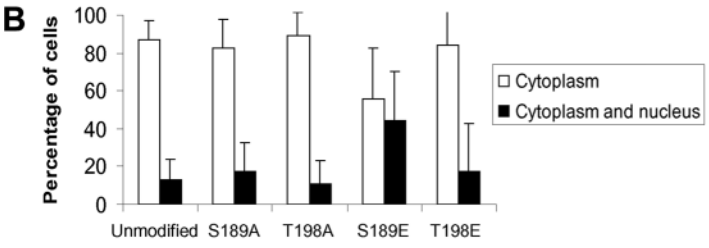


Fig. 4. Phosphorylation mimic at S189 and not T198 affects the localization of mSTI1-EGFP. (A) NIH3T3 fibroblasts were stably transfected with pB-mSTI1-EGFP or derivative plasmids pB-mSTI1(S189A)-EGFP, pB-mSTI1(T198A)-EGFP, pB-mSTI1(S189E)-EGFP and pB-mSTI1(T198E)-EGFP. Cells were fixed in 3.7% paraformaldehyde before mounting and visualization by confocal laser fluorescence microscopy. Scale bars, 10 μm. (B) Cells were transfected as above, and cells demonstrating cytoplasmic fluorescence or nuclear and cytoplasmic fluorescence were quantified. The error bars represent standard deviations.

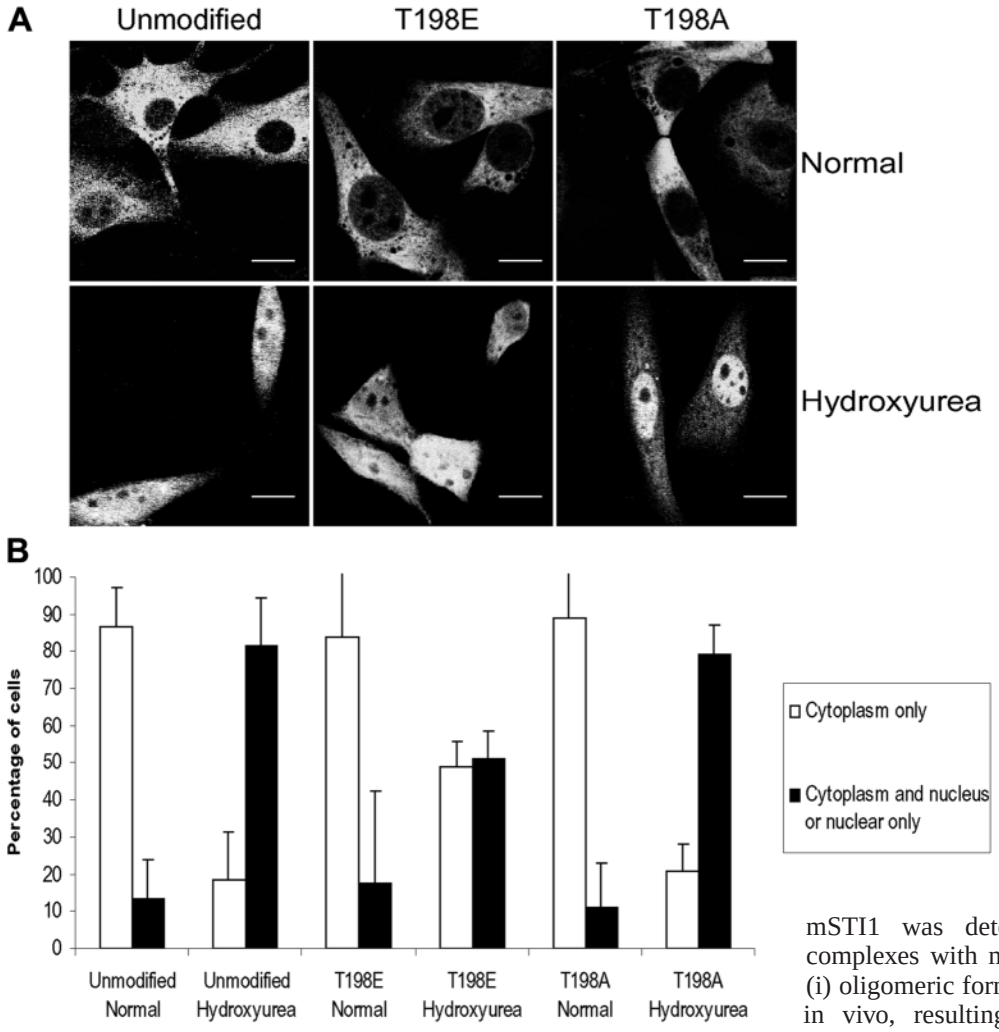


Fig. 5. Phosphorylation mimic and phosphorylation site removal at T198 affects the localization of mSTI1-EGFP under G1/S-phase arrest. (A) NIH3T3 fibroblasts were stably transfected with pB-mSTI1-EGFP, derivative plasmids pB-mSTI1(T198E)-EGFP or with pB-mSTI1(T198A)-EGFP. Cells were treated with hydroxyurea (10 mM) for 10 hours and fixed in 3.7% paraformaldehyde before mounting and visualization by confocal laser fluorescence microscopy. Scale bars, 10 μm. (B) Cells were transfected as above, and cells demonstrating cytoplasmic fluorescence or nuclear and cytoplasmic fluorescence were quantified. The error bars represent standard deviations.

(13%) under normal conditions (Fig. 4A,B). By contrast, although similar proportions of cells expressing mSTI1-EGFP and mSTI1(T198A)-EGFP showed cytoplasmic and nuclear localization of expressed protein, a greater nuclear accumulation of mSTI1(T198A)-EGFP was observed after hydroxyurea treatment (Fig. 5A,B). The removal of this cdc2 kinase phosphorylation site therefore promoted nuclear localization of mSTI1-EGFP under G1/S-phase arrest conditions. These results further confirm the data using the cdc2 inhibitor.

mSTI1-EGFP and its modified derivatives interacted with mSTI1, Hsp70 and Hsp90

Previously we showed that fusion of mSTI1 to GST and the amino acid substitutions S189A and T198A did not affect the functional interaction of mSTI1 with heat-shock proteins (Lässle et al., 1997; Longshaw et al., 2000). Using anti-GFP antibodies, we immunoprecipitated mSTI1-EGFP and its modified derivatives from lysates of transfected NIH3T3 cells. All the mSTI1-EGFP fusion proteins were found to be associated with Hsp70 and Hsp90 (Fig. 6). Hsp70 and Hsp90 could not be detected in immunoprecipitates from mock-transfected or EGFP-expressing cells. In addition, Hsp70 and Hsp90 were not detected in immunoprecipitations when using antibodies not specific for GFP. Taken together, these data suggested that mSTI1-EGFP and all its derivatives used in this study are functionally equivalent with respect to their interactions with Hsp70 and Hsp90. Interestingly, endogenous mSTI1 was detected in the immunoprecipitated complexes with mSTI1-EGFP, suggesting that either (i) oligomeric forms (possibly dimers) of mSTI1 exist in vivo, resulting in mSTI1/mSTI1-EGFP hetero-oligomers when exogenous mSTI1-EGFP is present, or (ii) more than one subunit of mSTI1 can separately bind within these chaperone complexes resulting in complexes that contain endogenous mSTI1 and exogenous mSTI1-EGFP.

Threonine 198 was the major but not the only site phosphorylated in vitro by cdc2 kinase

Previously, we reported the in vitro phosphorylation of mSTI1 by cdc2 kinase at T198 using recombinant tagged mSTI1 protein in cdc2 kinase assays (Longshaw et al., 2000). To test the possible existence of cdc2 kinase phosphorylation sites other than T198, we used untagged recombinant mSTI1 and mSTI1(T198A) in cdc2 kinase assays, and incubated for 3 hours and 9 hours to enhance any low levels of phosphorylation at sites other than T198. These longer incubations detected a low level of phosphorylation in the mSTI1(T198A) reaction that was above that detected for the autophosphorylation control (Fig. 7). Therefore, cdc2 kinase was phosphorylating another site, which we speculated to be T332, the only other site in mSTI1 that conforms to the cdc2 kinase consensus sequence. This region resembles another putative CcN motif (amino acids 326-351) with a putative CKII site at position S326, a putative cdc2 kinase site at position T332 and a putative bipartite NLS at positions 337-351. Removal of the T332 site was performed to produce mSTI1(T198A,T332A).

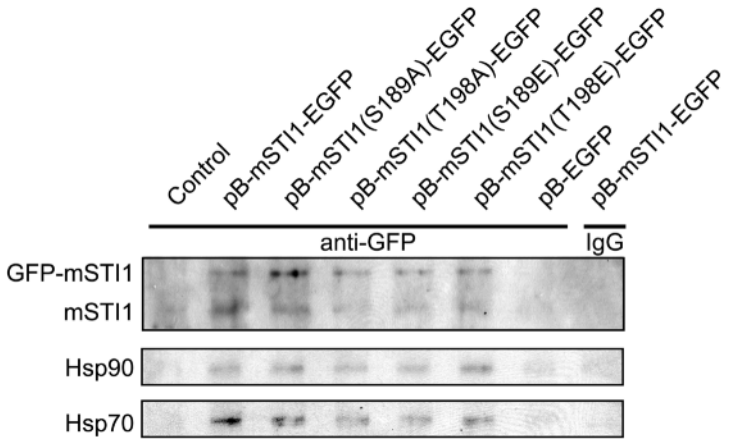


Fig. 6. mSTI1-EGFP and its modified derivatives can interact with mSTI1, Hsp70 and Hsp90. Cells were transfected with pB-mSTI1-EGFP or derivative plasmids pB-mSTI1(S189A)-EGFP, pB-mSTI1(T198A)-EGFP, pB-mSTI1(S189E)-EGFP, pB-mSTI1(T198E)-EGFP or pB-EGFP. EGFP chimeric proteins were immunoprecipitated from transfected and control untransfected cell lysates with antibodies against GFP (anti-GFP) or non-immune IgG (IgG) and subjected to western blotting, as indicated. The mSTI1-EGFP and mSTI1 proteins were detected with a polyclonal antibody raised against GST-mSTI1 (Lässle et al., 1997). Hsp90 was detected with monoclonal antibody sc31119 and Hsc70 and Hsp70 were detected with monoclonal antibody BRM-22. The mSTI1, Hsp90 and Hsp70 proteins all specifically immunoprecipitated with the mSTI1-EGFP chimeric proteins.

A lower level of phosphorylation was observed for the double mutant, suggesting that T332 might also be phosphorylated by cdc2 kinase. Some residual phosphorylation was still observed (Fig. 7), however, indicating that there might be another, as yet unidentified, cdc2 kinase phosphorylation site in mSTI1 that did not map to T198 or T332. The presence of multiple in vitro cdc2 kinase phosphorylation sites might explain the spectrum of mSTI1 isoforms observed in vivo (Lässle et al., 1997) (data not shown), although T198 appears to be the major in vitro cdc2 kinase site and can influence the cytoplasmic/nuclear shuttling of the mSTI1.

Discussion

Chaperones and heat-shock proteins are being recognized as increasingly important in cell signalling events because of their association with cell cycle components, regulatory proteins and members of the mitogenic signal cascade (Helmbrecht et al., 2000). We previously showed that the co-chaperone mSTI1 was phosphorylated in vitro by the cell cycle kinases, cdc2 and CKII, and that the sites of phosphorylation (T198 and S189, respectively) were close to a putative NLS (Longshaw et al., 2000). These data indicated that the cellular function of mSTI1 might be linked to cell cycle events. Here, we have provided evidence for the first time that mSTI1 has a functional NLS and that its localization was affected by cdc2 kinase and was cell-cycle dependent.

The mSTI1-EGFP expression system readily facilitated the analysis of the effects on localization of defined amino acid substitutions at the putative cell cycle kinase phosphorylation sites. Because mSTI1-EGFP and its modified derivatives were all found in complexes with Hsp70 and Hsp90 by immunoprecipitation, the effects of the amino acid substitutions on localization were unlikely to be the result of changes in Hsp70-mSTI1 or Hsp90-mSTI1 association. Interestingly, endogenous mSTI1 was also found in the immunoprecipitated complexes. We have previously shown that mSTI1 can exist as a dimer under certain conditions (van der Spuy et al., 2000; van der Spuy et al., 2001), and others have shown that yeast STI1 is able to dimerize (Prodromou et al., 1999). Therefore, for our mSTI1-EGFP studies, there is the potential that endogenous mSTI1 subunits have oligomerized with mSTI1-EGFP subunits. Such oligomerization could have potentially moderated the effects on localization of the amino acid substitutions (S189A, S189E, T198A and T198E). Nevertheless, changes in localization were detected using modified derivatives of mSTI1-EGFP allowing us to probe the mechanism of mSTI1 nuclear-cytoplasmic shuttling.

To test the functionality of the potential NLS of mSTI1, mouse NIH3T3 cells were transfected with constructs that expressed EGFP and NLS^{mSTI1}-EGFP. There was a high incidence of nuclear accumulation of NLS^{mSTI1}-EGFP relative to the distribution of EGFP that was both cytoplasmic and nuclear. This suggested that the putative NLS of mSTI1 was functional and that mSTI1 might accumulate in the nucleus under certain conditions. Interestingly, endogenous mSTI1 and exogenously expressed mSTI1-EGFP were found to accumulate in the nucleus when nuclear export was inhibited with leptomycin B. Therefore, we propose that the subcellular distribution of mSTI1 is a dynamic balance of nuclear import and export processes, the fulcrum of which might be shifted under different conditions. The formal demonstration of the functionality of the putative NLS would require mutagenesis of the NLS to produce mutant versions that are capable of associating with Hsp70 and Hsp90 but are disrupted with respect to nuclear localization. We are currently conducting studies such as these to comprehensively map the boundaries of the NLS.

It is interesting to note that the Hsp90-binding TPR domain of mSTI1 (TPR2A) overlaps with the predicted NLS. Based on the structure of the TPR2A domain of Hop in complex with the Hsp90 C-terminal MEEVD peptide (Scheufler et al., 2000), the conserved residues K229 and N233 make contact with the

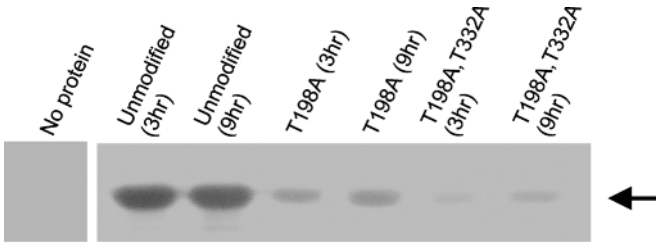


Fig. 7. mSTI1 is phosphorylated in vitro at multiple sites by cdc2 kinase. In vitro cdc2 kinase assays were incubated for 3 hours and 9 hours using purified recombinant mSTI1 and its modified derivatives (5 μM). The phosphorylated proteins were resolved by SDS-PAGE and the radioactive phosphorylation in the dried gel was detected by autoradiography.

terminal aspartic acid residue (D^0) of the peptide. Furthermore, Y236 makes contact with E^{-3} and M^{-4} of the Hsp90 peptide. K229, N233 and Y236 are located within the mSTI1 NLS spacer region. Hsp90 and karyopherin-α are relatively large proteins (180 kDa and 58 kDa, respectively), and it is therefore proposed that mSTI1 might be sterically restricted from simultaneously binding Hsp90 and karyopherin-α, owing to the close proximity of their respective binding sites. This alternate binding of Hsp90 or karyopherin-α might have mechanistic implications for the formation of the Hsp70-mSTI1-Hsp90 chaperone complex and its localization. Furthermore, conformational changes induced by phosphorylation at various sites proximal to these overlapping binding sites might allow preferential binding of either Hsp90 or karyopherin-α. Phosphorylation of sites within the CcN motif might therefore not only be a level of regulation of the import kinetics of the NLS, but also of the assembly of the Hsp70-mSTI1-Hsp90 chaperone complex. We are currently investigating both of these possibilities.

We conducted a set of experiments to determine whether or not CKII had a role in the regulation of the localization of mSTI1. Treatment of cells with DRB did not result in any significant change in the localization of endogenous mSTI1 or exogenously expressed mSTI1-EGFP. This suggested that inactivation of CKII did not change the distribution of mSTI1, implying that, under normal conditions, CKII was not important in maintaining the largely cytoplasmic localization of mSTI1. This conclusion was supported by the finding that an alanine substitution at the proposed CKII phosphorylation site (S189) had no effect on the localization of mSTI1. By contrast, mimicking of phosphorylation by glutamic acid substitution at the proposed CKII phosphorylation site promoted a nuclear localization of mSTI1-EGFP. This suggested an enhancing effect of a negative charge at the S189 site on mSTI1-EGFP nuclear import under normal conditions. In addition, these data provided indirect evidence that S189 was an in vivo site of phosphorylation and that phosphorylation at this site enhanced the nuclear accumulation of mSTI1.

In contrast to CKII, our experiments suggested that cdc2 kinase might regulate mSTI1 localization at the level of cytoplasmic retention. Endogenous mSTI1 and exogenously expressed mSTI1-EGFP accumulated in the nucleus when cells were arrested in G1/S phase and when cdc2 kinase was inactive. In addition, mSTI1(T198E)-EGFP was found to have a lower incidence of nuclear accumulation than mSTI1-EGFP under conditions of G1/S-phase arrest, whereas there was an increased incidence of nuclear accumulation of mSTI1-EGFP(T198A) under the same conditions. These amino acid substitution results suggested that a negative charge at the predicted cdc2 kinase phosphorylation site inhibited nuclear accumulation or, conversely, promoted cytoplasmic retention. Taken together, these results suggested that mSTI1 was retained in the cytoplasm under normal conditions possibly through phosphorylation at the cdc2 kinase phosphorylation site (T198). This would mean that any subsequent recruitment of mSTI1 to the nucleus at G1/S-phase arrest would require the generation of unphosphorylated or dephosphorylated mSTI1. Therefore, the mechanism of nuclear translocation might involve: (i) inactivation of cdc2 kinase, so that increased levels of newly synthesized unphosphorylated mSTI1 accumulate as a result of protein turnover; and/or (ii) active dephosphorylation of existing mSTI1 at the cdc2 kinase phosphorylation site by activation/upregulation of a specific phosphatase.

The mSTI1 protein might be recruited to the nucleus of G1/S-phase-arrested cells or during S phase in concert with its chaperone partners, Hsp70 and Hsp90. Although the co-chaperone function of mSTI1 within the cytoplasm has been extensively studied, the role of mSTI1 within the nucleus remains to be elucidated. We propose that the basic role of mSTI1 as a co-chaperone for Hsp70 and Hsp90 probably applies within the nucleus. However, this does not exclude the possibility that mSTI1 has other specialized activities within the nucleus. There is evidence that Hsp70 is involved in chromosomal DNA replication (Milarski and Morimoto, 1986; Hang and Fox, 1995), and Hsp90 has been implicated in the activation of telomerase and the replication of chromosome ends (Forsythe et al., 2001; Grandin and Charbonneau, 2001). Hsp70 has been shown to translocate to the nucleus during S phase and during recovery of S phase after stress, suggesting a role for Hsp70 during activation and/or recovery of DNA replication (Milarski and Morimoto, 1986; Suzuki and Watanabe, 1992; Zeise et al., 1998). In addition, it was recently shown that STI1/Hop and Hsp70 are components of an S-phase transcription activator complex (Zheng et al., 2003). It remains to be determined the extent to which mSTI1 is involved in Hsp70/Hsp90-directed chaperone activities within the nucleus. Because stresses such as heat shock perturb the cell cycle, causing arrest at the G1/S and the G1/M transitions (Kühl et al., 2000), we are currently studying the effects of heat shock on the localization of mSTI1.

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