Multisite Phosphorylation and the Nuclear Localization of Phosphatase Inhibitor 2-Green Fluorescent Protein Fusion Protein during S Phase of the Cell Growth Cycle*

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Human phosphatase inhibitor 2 (Inh2) is a phosphoprotein that complexes with type 1 protein phosphatase, and its expression peaks during S phase and mitosis during the cell cycle. Localization of Inh2 was visualized in Hs68 human fibroblasts by fusing Inh2 to green fluorescent protein (GFP). During G₁ phase, Inh2-GFP was localized in the cytoplasm, and as cells progressed into S phase Inh2-GFP accumulated in the nucleus. Known phosphorylation sites of Inh2 at Thr-72, Ser-86, and Ser-120/121 were each replaced with alanine. None of the mutated Inh2-GFP proteins accumulated in the nucleus during S phase, indicating that all of these phosphorylation sites were required. Mutation of two lysine residues in a putative nuclear localization sequence in Inh2 also prevented the Inh2-GFP fusion protein from accumulating in the nucleus during S phase. Recombinant Inh2 was phosphorylated by kinases in cytosols prepared from G₁ and S phase cells. The amount of Inh2 kinase attributed to casein kinase 2, based on inhibition by heparin, increased 2.6-fold from G₁ to S phase. In the cell cycle. Localization of Inh2 was visualized in human fibroblasts by fusing Inh2 to green fluorescent protein (GFP). During G₁ phase, Inh2-GFP was localized in the cytoplasm, and as cells progressed into S phase Inh2-GFP accumulated in the nucleus. Known phosphorylation sites of Inh2 at Thr-72, Ser-86, and Ser-120/121 were each replaced with alanine. None of the mutated Inh2-GFP proteins accumulated in the nucleus during S phase, indicating that all of these phosphorylation sites were required. Mutation of two lysine residues in a putative nuclear localization sequence in Inh2 also prevented the Inh2-GFP fusion protein from accumulating in the nucleus during S phase. Recombinant Inh2 was phosphorylated by kinases in cytosols prepared from G₁ and S phase cells. The amount of Inh2 kinase attributed to casein kinase 2, based on inhibition by heparin, increased 2.6-fold from G₁ to S phase. In addition, kinases in G₁, versus S phase cytosols produced distinct Inh2 phosphopeptides. The results indicate that changes in phosphorylation of Inh2 are involved in intracellular redistribution of the protein during the cell cycle.

Human phosphatase inhibitor-2 (Inh2)¹ is a 23-kDa heat-stable protein first identified as a phosphatase inhibitor protein in rabbit skeletal muscle (1). Rabbit Inh2 was purified to homogeneity, (2, 3) and the amino acid sequence was determined as 204 residues (4). The cDNA sequences of rabbit skeletal muscle Inh2 (5, 6), rabbit liver Inh2 (5), and human Inh2 (7) have been determined. These amino acid sequences are over 90% identical. The GLC8 gene in Saccharomyces cerevisiae encodes a protein with some similarity to Inh2, but only 25% of the amino acid sequence is identical (8–10).

Inh2 forms a stable heterodimer with the catalytic subunit of type 1 protein phosphatase (PP1) (11–15) that has been called MgATP-dependent phosphatase. It is activated by reaction with glycogen synthase kinase 3 in the presence of MgATP (12, 16–19), which produces a transient phosphorylation of Thr-72 in Inh2. Both cyclin B-cdc2 (20) and mitogen-activated protein kinase (21) can also phosphorylate Thr-72 of Inh2 in biochemical assays. The phosphorylation sites found by protein sequencing were Ser-86, Ser-120, and Ser-121, all phosphorylated by casein kinase 2 (CK2) (22). Prior phosphorylation of Inh2 by CK2 enhances phosphorylation by glycogen synthase kinase 3 (5, 12). Inh2 also can be phosphorylated on a tyrosine residue by purified insulin receptor, with loss of its inhibitory activity toward PP1 (23). Phosphoamino acid analysis of Inh2 from mouse diaphragm (24) and rat fat cells (25) showed predominantly phosphoserine and only trace amounts of phosphothreonine.

The function of Inh2 and the role of its phosphorylation in living cells remains unsettled. One proposal is that MgATP-dependent phosphatase is a significant cytoplasmic form of PP1. Results using PP1 expressed in bacteria led to another proposal that Inh2 functions as a molecular “chaperone” for folding newly synthesized PP1 into a biologically active conformation (26, 27). On the other hand, the amount of Inh2 protein and heat-stable inhibitory activity against PP1 were found to oscillate during the cell cycle in rat embryo fibroblasts, peaking during S phase and mitosis (28, 29), suggesting a possible role for Inh2 in the cell division cycle. A similar cell cycle-dependent change in the amount of the yeast GLC8 protein has been reported (8).

The green fluorescent protein (GFP) of jellyfish Aequorea victoria (30, 31) has emerged as a unique tool for examining intracellular phenomena in living cells. Because GFP possesses an intrinsic fluorescence that does not require other cofactors and because the S65T mutant of GFP shows an enhanced brightness compared with the wild type protein, fusion proteins with GFP/S65T provide a powerful system to analyze protein expression and distribution in living cells (32–34). In the present study, we used a Inh2-GFP fusion protein to examine intracellular localization of Inh2 during G₁ and S phases of the cell cycle.

MATERIALS AND METHODS

Cell Culture—Human foreskin fibroblasts (HS68 cells) and SV40-transformed African green monkey kidney cells (COS-7) were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) plus 2 mM glutamine. Cell growth was synchronized in serum-free DMEM for 36–48 h, followed by addition of 10% FCS. Addition of 1–2 mM hydroxyurea was used to arrest cells near G₁/S. For fluorescence microscopy to detect GFP protein, cells were cultured in phenol
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RESULTS

Intracellular Localization of Inh2-GFP during G1 and S Phase—The intracellular localization of an Inh2-GFP fusion protein was examined in HS68 cells made quiescent by serum deprivation for 36–48 h and then stimulated into synchronous growth by serum refeeding. To define the time of S phase, cells were pulse-labeled for 4-h intervals with [3H]thymidine. Fig. 1A shows a sharp peak of DNA synthesis at 21 h. As an alternative method for synchronization of cell growth, we used hydroxyurea to block cells near G1/S. After washout of hydroxyurea, incorporation of [3H]thymidine could be detected as early as 30 min, and it reached a peak at 2 h (Fig. 2A). At the peak of S phase, more than 70% of the cells were immunostained for incorporation of bromodeoxyuridine using either method of synchronization (data not shown). The results of both analyses were consistent and established two alternative methods for the synchronization of HS68 cells in S phase.

GFP fluorescence was detected in approximately 5% of cells transfected with an Inh2-GFP expression vector. Transfection efficiency for HS68 cells was limited by the serum starvation protocol used to provide synchronous growth of the cells. After various trials, it was found that transfections could be done during the time period just prior to stimulation of serum. After serum stimulation, Inh2-GFP protein was predominantly cytoplasmic up to 12 h, corresponding to the G1 phase of growth (Fig. 1C, panel a). However, Inh2-GFP protein accumulated in the nucleus as these cells progressed from G1 to S phase (Fig. 1C, panel b). At 21 h, when 70% of the cells were in S phase as shown by bromodeoxyuridine staining, nuclear translocation-
positive cells reached 68 ± 15%, expressed as a N/C index of 0.68 (Fig. 1B, closed circles). Most of the cells transfected with GFP alone as a control exhibited green fluorescence uniformly throughout the cell (Fig. 1C, panel c), and these cells had a low N/C index during S phase (Fig. 1B, open circles). In separate experiments, at 2.5 h after hydroxyurea release (that is, during S phase [Fig. 2A]), Inh2-GFP protein accumulated in the nucleus, again expressed as a N/C index > 0.6 (Fig. 2B, closed circles), whereas control cells expressing GFP itself did not show accumulation of GFP in the nucleus (Fig. 2B, open circles). The results show that the Inh2-GFP protein was predominantly cytoplasmic during G1 phase and that it accumulated in the nucleus during S phase. Both methods for synchro-
nization of cell growth gave the same percentage of green fluorescent cells exhibiting nuclear localization of the Inh2-GFP fusion protein (N/C index), and this agreed exactly with the percentage of cells in the entire culture that were positive for bromodeoxyuridine staining.

As an additional control experiment, we examined whether transient expression of Inh2-GFP or GFP itself affected entry into S phase. FACScan analysis by propidium iodide staining showed the distribution of mock-transfected cells (Fig. 3A, broken line) and 2 h after washout (solid line); B, cells expressing GFP itself, 2 h after hydroxyurea washout; C, cells expressing Inh2-GFP, 2 h after hydroxyurea washout. The abscissa shows relative DNA content, and the ordinate shows the number of cells. Expression of GFP or Inh2-GFP did not affect entry into S phase following arrest with hydroxyurea.

**FIG. 3.** Expression of Inh2-GFP does not alter cell cycle progression. COS-7 cells expressing either GFP itself or Inh2-GFP were analyzed using FACScan. Cells were transfected and growth arrested by addition of 2 mM hydroxyurea to the medium for 20 h. Cells were collected, sorted based on GFP fluorescence as described under “Materials and Methods,” and then analyzed using propidium iodide staining. The following populations of cells were compared: A, mock-transfected cells, blocked with hydroxyurea (broken line) and 2 h after washout of hydroxyurea (solid line); B, cells expressing GFP itself, 2 h after hydroxyurea washout; C, cells expressing Inh2-GFP, 2 h after hydroxyurea washout. The abscissa shows relative DNA content, and the ordinate shows the number of cells. Expression of GFP or Inh2-GFP did not affect entry into S phase following arrest with hydroxyurea.

**Fig. 4.** Effects of mutations on intracellular distribution of Inh2-GFP fusion proteins. HS68 cells grown on a coverglass were transfected with plasmids encoding wild type or mutated Inh2-GFP fusion proteins and then synchronized with hydroxyurea as described in the legend to Fig. 2. The living cells were examined using a fluorescence microscope 2.5 h after washout of hydroxyurea. The results are shown as the mean ± S.D. (n = 5) of the N/C index defined under “Materials and Methods.” Schematic representation of the Inh2-GFP constructs is shown under the graph. The position of mutations of the Inh2-GFP fusion proteins is given as one-letter code in the box. Numbers in the lower panel correspond to column numbers in the upper panel.

did not affect cell cycle progression into S phase following hydroxyurea blockade.

**Mutations to Define Requirements for Nuclear Accumulation of Inh2 during S Phase**—The known phosphorylation sites in Inh2 were mutated to alanines using megaprimer polymerase chain reaction, creating T72A-Inh2, S86A-Inh2, and S120A/S121A-Inh2, which were expressed as GFP fusion proteins in HS68 cells. In contrast to the wild type Inh2-GFP (Fig. 4, column 2), none of the point mutants accumulated in the nucleus during S phase (Fig. 4, columns 3–5). The N/C index <0.2 was the same as that for GFP itself (Fig. 4, column 1). The total number of green fluorescent cells was the same for GFP, for mutated Inh2-GFP, and for wild type Inh2-GFP, and the results were replicated in three to five independent experiments. Thus, mutation of any one of the known phosphorylation sites in Inh2 altered localization of the Inh2-GFP fusion protein in living cells during S phase.

Inh2 has two clusters of basic amino acid residues within the sequence 134–147 (REKKRQFEMKRKLH. The lysine residues at positions 143 and 145 were mutated to alanines, giving a K143A/K145A mutant of Inh2 (Inh2-KK/AA). rInh2 with lysines 143 and 145 mutated to non-basic amino acids had inhibitory specific activity toward PP1 that was identical to the wild type Inh2 protein (data not shown). Inh2-KK/AA-GFP fusion protein expressed in HS68 cells did not become concentrated within the nucleus during S phase (Fig. 4, column 6). There was no difference in the number of cells expressing Inh2-KK/AA-GFP, wild type Inh2-GFP, or GFP itself.
of lysines at positions 143 and 145 was like mutation of phosphorylation sites in Inh2; i.e. it prevented nuclear localization of the Inh2-GFP fusion protein in living cells during S phase.

Phosphorylation of Inh2 and Inh2-GFP in Living Cells—Inh2 is phosphorylated by multiple protein kinases, providing a potential mechanism for regulating its localization. Antibodies against Inh2 or GFP were not effective at immunoprecipitation in our hands, preventing analysis of phosphorylation by direct recovery of endogenous Inh2 or transiently expressed Inh2-GFP. As an alternative, to produce enough protein for analysis of phosphorylation, COS-7 cells were transfected with plasmids encoding epitope-tagged FLAG-GFP, FLAG-Inh2, or FLAG-Inh2-GFP. Cells were synchronized into S phase by hydroxyurea block/release and metabolically labeled with 32P. As shown in Fig. 5, FLAG-Inh2-GFP and FLAG-Inh2 were32P-labeled (Fig. 5A, lanes 2 and 3), whereas the FLAG-GFP protein was not. FLAG-Inh2-GFP and FLAG-Inh2 were phosphorylated to approximately the same specific radioactivity (FLAG-Inh2-GFP/FLAG-Inh2 = 0.8), calculated by densitometry. We concluded that the sites in the Inh2 portion, not in the GFP portion, of the fusion protein were phosphorylated in living cells. Presumably, the same sites were phosphorylated in FLAG-Inh2-GFP and FLAG-Inh2. Acid hydrolysis and phosphoamino acid analysis revealed only 32P-labeled Ser in these proteins (data not shown).

Phosphorylation of Thr-72 in Inh2 in Living Cells during S Phase—To provide evidence that Thr-72, which was required for nuclear accumulation during S phase (Fig. 4), was phosphorylated in living cells, COS-7 cells were transfected with plasmids for FLAG-Inh2, FLAG-T72A-Inh2, FLAG-S86A-S120A-S121A-Inh2 (triple mutant), and FLAG-T72A-S86A-S120A-S121A-Inh2 (quadruple mutant). Cells were synchronized into S phase and metabolically labeled with 32PO4 as described above. Immunoprecipitates from these cells using anti-FLAG M2 antibody were subjected to SDS-PAGE, Coomassie Blue staining, and autoradiography. As shown in Fig. 6A, FLAG-Inh2 and FLAG-T72A-Inh2 were robustly phosphorylated (lanes 2 and 3). In comparison, the triple mutant FLAG-S86A-S120A-S121A-Inh2 was also 32P-labeled, but at a much lower level (lane 4). The quadruple mutant FLAG-T72A-S86A-S120A-S121A-Inh2 had 32P labeling even lower than that of the triple mutant FLAG-S86A-S120A-S121A-Inh2. This difference in 32P labeling between the triple and quadruple mutant proteins was attributed to phosphorylation of T72. Interestingly, even the quadruple mutant Inh2 was radiolabeled (Fig. 6A, lane 5), showing that there are other sites in Inh2 phosphorylated in these cells. Coomassie Blue staining was used to measure the recovery of various FLAG-Inh2 proteins (Fig. 6B). Although the same amount of antibody was used for each sample, as seen from staining for the heavy chain at the top of each lane, there were different amounts of FLAG-Inh2 recovered in each sample. In the Coomassie-stained gel, differences in electrophoretic mobility of the various Inh2 proteins were evident. The single mutation of T72 resulted in increase in mobility relative to wild type (Fig. 6B, lane 2 versus lane 3). The triple and quadruple mutants migrated in the same way (Fig. 6B, lanes 4 and 5), and this was different from the wild type and T72A forms of Inh2. The mobility in SDS-PAGE is another indication of phosphorylation of these sites in Inh2. The results are further evidence for multisite phosphorylation of Inh2 at T72, S86, S120, and S121 in living cells during S phase.

Differential Phosphorylation of Recombinant Inh2 by Kinases in the Cytosol from G1 and S Phase Cells—Inh2 kinase activity in cytosols prepared from G1 versus S phase cells was assayed with rInh2 as an exogenous substrate. As shown in Fig. 7A, rInh2 was phosphorylated by kinases present in the cytosol of HeLa fibroblasts. Cytosols prepared from S phase cells (Fig. 7A, lane 4) had a specific activity (32P labeling/µg of cytosol protein) that was 1.6 times higher than that of cytosols from G1 phase cells (Fig. 7A, lane 1). Rabbit Inh2 is known to be phosphorylated at S66, S120, and S121 by CK2, so we added 1 µg of heparin, an inhibitor of CK2 (42), to the 60-µl reaction mixtures. At this dosage, heparin strongly inhibited the phosphorylation of rInh2 (Fig. 7A, compare lanes 1 versus 2 and lanes 4 versus 5), reducing labeling by 40% using G1 or by 60% using S phase cytosol as a source of kinase (quantitation done by scintillation counting of the excised proteins). We calculated a 2.6-fold increase from G1 to S phase in the heparin-sensitive Inh2 kinase activity, attributed to CK2.

To examine the sites of phosphorylation in rInh2, the radiolabeled samples shown in Fig. 7A, lanes 1 and 4, were excised from the gel and subjected to two-dimensional tryptic phosphopeptide mapping as described under “Materials and Methods.”

FIG. 5. In vivo phosphorylation of Inh2-GFP fusion proteins during S phase. COS-7 cells were transfected and synchronized as described in the legend to Fig. 3. Cells were labeled with [32P]orthophosphate for 1 h in phosphate-free DMEM plus 10% dialyzed FCS. Anti-FLAG M2 immunoprecipitates were separated by SDS-PAGE and transferred to the filter. Lane 1, FLAG-Inh2-GFP; lane 2, FLAG-GFP; lane 3, FLAG-Inh2. A, immunoblotting using anti-FLAG M2 monoclonal antibody.

FIG. 6. In vivo phosphorylation of wild type and mutated Inh2 proteins during S phase. A, phosphorylation of wild type and mutated FLAG-Inh2 proteins was performed in COS-7 cells labeled with [32P]orthophosphate for 1 h in phosphate-free DMEM plus 10% dialyzed FCS. Anti-FLAG M2 immunoprecipitates were separated by SDS-PAGE and transferred to the filter. Lane 1, FLAG alone; lane 2, FLAG-Inh2; lane 3, FLAG-T72A-Inh2; lane 4, FLAG-S86A-S120A-S121A-Inh2; lane 5, FLAG-T72A-S86A-S120A-S121A-Inh2.
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This study examined the intracellular localization of Inh2 in living cells during G1 and S phases of the cell cycle using an Inh2-GFP fusion protein. Accumulation of Inh2-GFP from the cytoplasm into the nucleus occurred during S phase. GFP itself, expressed as a control, remained uniformly distributed in the cell throughout G1 and S phase. Mutations to eliminate phosphorylation of Inh2 at either Thr-72, or Ser-86, or Ser-120 and Ser-121 abolished nuclear localization during S phase. Differences in amino acid residues at positions immediately adjacent to a phosphorylation site (e.g. C58 in human versus Y85 in rabbit) do not affect nuclear import of Inh2, supporting the idea that the multisite phosphorylation of Inh2 is required for its cell cycle-dependent localization.

Phosphorylation of Inh2 is cooperative, or “synergistic,” because reaction with CK2 at Ser-86 potentiated phosphorylation of Thr-72 by glycogen synthase kinase 3, making phosphorylation of Thr-72 highly sensitive to the extent of prior phosphorylation by CK2. Different proline-directed protein kinases, namely glycogen synthase kinase 3, CDK2, and mitogen-activated protein kinase, can phosphorylate Thr-72. Therefore, it is possible that mutation of Ser-86 prevented nuclear localization indirectly by interfering with efficient phosphorylation of Thr-72. We present evidence that Thr-72 was phosphorylated in living cells even in the triple mutant S86/120/121A. One might suspect, therefore, that the failure of the S86A mutant of Inh2-GFP to localize in the nucleus indicates a function for this site beyond simply promoting phosphorylation of Thr-72. In addition, the CK2 phosphorylation sites at S120/121 themselves might facilitate nuclear accumulation, parallel to the case of SV40 T antigen (see below). It seems that all of these phosphorylation sites in Inh2 contribute to and are required for nuclear localization during S phase.

Cell cycle-dependent nuclear localization of Inh2 also depended on a basic sequence resembling a NLS. Because the molecular size of Inh2-GFP is 63 kDa, it seems unlikely that the distribution of this fusion protein was a result of simple diffusion between cytoplasm and nucleus (43, 44). At residues 134–147 in Inh2, there is a sequence of two clusters of basic amino acids in a region of Inh2 that is related in sequence to c-fos (5, 22). Mutation of lysines 143 and 145 abolished nuclear localization of Inh2 during S phase. As a transcription factor, c-fos surely is imported into the nucleus, but its NLS has not been functionally defined. Maybe Inh2 and c-fos are similar in this regard, or alternatively, if a given signal is removed from Inh2 it is possible that it can no longer be recognized by the cell for localization. The NLS of Inh2 appears to be functionally defined. Maybe Inh2 and c-fos are similar in sequence in the region used as their NLS.

There are several examples of nuclear import of proteins mediated by a NLS and regulated by phosphorylation. Undoubtedly the best known example is that of SV40 T antigen, which has a NLS with the sequence PKKKRKV (45). When this sequence is fused or conjugated to other proteins, it results in their accumulation in the nucleus. However, it has not been well appreciated that the rate of nuclear import mediated by this NLS is controlled by phosphorylation of two serine sites approximately 20 residues to the N-terminal side (46). Phosphorylation of these serines by CK2 accelerated by 30-fold the rate of nuclear import of a SV40 T antigen-β galactosidase fusion protein (47). The spacing of the dual CK2 Ser phosphorylation sites and basic residues of the putative NLS in Inh2 matches that in large T antigen, so similar mechanisms may govern localization of these proteins. Phosphorylation of cyclin B also results in nuclear localization, which is critical for its function at G2/M in the cell cycle (48). The sites in cyclin B under cell cycle control are thought to be phosphorylated by CK2 and mitogen-activated protein kinase, an interesting par-

Fig. 7. Phosphorylation of rInh2 by kinases in the cytosols from G1 and S phase cells. A, autoradiograph of 32P-phosphorylated rInh2 (2 μg; lanes 1, 2, 4, and 5) that was incubated without (lanes 1 and 4) or with (lanes 2 and 5) 1 μg of heparin for 1 h at 30 °C with cytosols (5 μg of protein) from synchronized HS68 cells (lanes 1–3; 8 h after serum stimulation; lanes 4–6; 21 h after serum stimulation) in the presence of [γ-32P]ATP. Lanes 3 and 6 are controls without added rInh2, to reveal phosphorylation of proteins in the cytosols. B, phosphopeptide mapping. The 32P-labeled rInh2 in lanes 1 and 4 of panel A and rInh2 labeled by purified CK2 were digested with trypsin, and peptides were resolved in two dimensions as described under “Materials and Methods.” a, phosphorylation by lysate of G1 cells, 8 h after serum stimulation; b, phosphorylation by lysate of G1 phase cells, 21 h after serum stimulation; c, phosphorylation by purified CK2. Sites of sample application are marked by x. Corresponding phosphopeptides are denoted with numbers.

ods.” As shown in Fig. 7B, panel a, there was one predominant tryptic phosphopeptide (peptide 1) plus several other phosphopeptides (peptides 2, 4, 5, and 6) recovered from rInh2 after labeling with G1 cytosol as a source of kinase. In contrast, rInh2 phosphorylated using cytosol from S phase cells (Fig. 7B, panel b) had only a trace of phosphopeptide 1 and instead was labeled with about equal intensity in five other phosphopeptides (peptides 2–6). Phosphopeptides 2, 4, and 5 matched those obtained from rInh2 phosphorylated with purified CK2 (Fig. 5B, panel c). Therefore, in S phase cells, CK2 accounted for most of the rInh2 kinase activity. There were two notable differences between the G1 and S phase phosphopeptide patterns. First, there was a kinase in G1 phase cytosols that produced phosphopeptide 1. This kinase and the site in Inh2 affected by phosphoamino acids at positions immediately adjacent to a phosphorylation site (e.g. C58 in human versus Y85 in rabbit) do not affect nuclear import of Inh2, supporting the idea that the multisite phosphorylation of Inh2 is required for its cell cycle-dependent localization.

2 J. Somers, N. J. C. Lamb, and D. L. Brautigan, unpublished data.
allel to Inh2. Other examples of phosphorylation-regulated nuclear import are the transcription factor SWI5 of *S. cerevisiae* and the v-Jun oncoprotein. Both proteins are localized in nuclei in their unphosphorylated forms. Phosphorylation of SWI5 by CDC28 kinase results in its displacement into the cytoplasm (49). Phosphorylation of v-Jun also is correlated to loss of nuclear localization (50). Thus, phosphorylation can be employed to enhance or to eliminate nuclear localization.

The Inh2 kinase activity in cytosols increased between G₁ and S phase. Most of this activity was potentially inhibited by heparin, a characteristic of CK2. The results fit together with the previous data showing that CK2 activity oscillates during the cell cycle, peaking at S phase (51). Inh2 phosphorylated by these S phase cytosols or by purified CK2 gave several of the same major phosphopeptides. This is consistent with Ser-86, Ser-120, and Ser-121, the sites in Inh2 phosphorylated by CK2, being phosphorylated during S phase. Peptides 3 and 6 were not produced by purified CK2, but were prominent in Inh2 phosphorylated by cytosols from S phase cells. The PhosPepSort2 program (GGC Computer Group) predicts this map location for phosphopeptides containing Thr-72, and because peptide 6 was produced by both G₁ and S phase cytosols, we suspect that Thr-72 is phosphorylated during throughout G₁ and S phases. A remaining issue is the identity of the site in tide 6 was produced by both G₁ and S phase cytosols, we Sort2 program (GCG Computer Group) predicts this map location not produced by purified CK2, but were prominent in Inh2

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