The Serum-Inducible Protein Kinase Snk Is a G1 Phase Polo-Like Kinase That Is Inhibited by the Calcium- and Integrin-Binding Protein CIB

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Abstract
Identified as an immediate-early transcript, the serum-inducible kinase Snk bears sequence homology with the polo-like kinases. Endogenous Snk was detected in early G1 in NIH 3T3 cells, and nascent Snk showed a half-life of about 15 min. The kinase activity of endogenous Snk was detected in G1. Substitution of Thr-236 with a glutamate residue increased Snk kinase activity by about 10-fold, whereas substitution of Lys-108 abolished its kinase activity. Disrupting the polo-box did not significantly change Snk kinase activity. A GFP-C-Snk fusion protein showed polo-box-dependent localization to the microtubule organizing center, and ectopic expression of Snk in COS-7 cells induced changes in cell morphology, depending on Snk kinase activity and the polo-box. The capacity of Snk to induce morphological changes was inhibited by the calcium- and integrin-binding protein CIB. CIB co-immunoprecipitated with Snk and inhibited the kinase activity of Snk, suggesting that CIB is a negative regulator for Snk kinase activity.

Introduction
The polo-like kinases (Plks) are a family of serine/threonine kinases closely related to polo of Drosophila melanogaster. These kinases have been identified in evolutionarily diversified eukaryotes, and they share a conserved NH2-terminal kinase domain and a homologous carboxyl terminus (1). A domain of about 30 amino acid residues in the COOH terminus is highly conserved among these kinases and thus dubbed the polo-box (1). The expression and activity of Plks are regulated in the cell cycle.

Many studies have been reported on the M phase Plks. These enzymes are highly expressed and active during G2-M of the cell cycle, and they are critical for events in mitosis and cytokinesis. The Xenopus Plk1 activates Cdc2 indirectly by phosphorylation and activation of Cdc25C (2–4). Plk1 and Plk3 have been reported to be involved in nuclear translocation of cyclin B1 (5), but a recent report argued that phosphorylation by Plk1 does not cause cyclin B1 to move into the nucleus (6). Mitotic Plks also appear to contribute to centrosome maturation, disintegration of the Golgi apparatus in mitosis (7–9), formation of bipolar spindles (3, 10, 11), and completion of cytokinesis (17–21). A role in regulating DNA replication was reported for the budding yeast CDC5 (22). As cells exit mitosis, mitotic Plks are destroyed by ubiquitin-mediated proteolysis (15, 23, 24). Because mitotic Plks are important regulators for mitosis, it is not surprising that their activity may be subjected to regulation by the DNA replication checkpoint (24–26). The activity of mitotic Plks is also regulated by phosphorylation, and a polo-like kinase kinase, xPlkk1, has been identified to phosphorylate and activate Plx1 (27).

In mammalian cells, two Plks, Fnk and Snk, were originally identified as immediate-early transcripts (28, 29). Further studies of Fnk show that the amount of Fnk increases from G1 to M phase, and Fnk is phosphorylated in mitosis, resulting in elevated kinase activity. Fnk is dephosphorylated at a later stage of mitosis and likely remains in the unphosphorylated form when cells reenter G1 (23). Thus, it appears that Fnk may function during entry into the cell cycle as well as in mitosis. Plk3 (Plk3), which is the human homologue of Fnk, phosphorylates Cdc25C in vitro, suggesting that it may play a role in entry into mitosis (30). Moreover, overexpression of Plk3 induced apoptosis and incomplete cytokinesis, and a EGFP-Plk3 localized to the cell midbody at the exit from mitosis (31). Fnk may also play a role in cell adhesion (32). Genes encoding possible homologues of Fnk and Snk are also found in Xenopus, and they are involved in oocyte maturation (33). In Caeorhabditis elegans, three cDNA sequences were found to contain the polo-box (34, 35). One of them appears to be a homologue of mitotic Plks, whereas the functions of the other two remain unclear (36).

In contrast to studies on the other Plks, the expression of Snk and its kinase activity in the cell cycle have not been reported. One study reported that Snk, as well as Fnk, is expressed in rat brain tissues, and their expression appears to be regulated by...
neuronal activities (37). Our results reported here describe the expression and activity of Snk in cultured mammalian cells. We also describe a functional interaction between Snk and the calcium- and integrin-binding protein CIB.

**Results**

*The Expression of Snk in the Cell Cycle*

The deduced amino acid sequence of Snk suggested that it belongs to the family of Plks (29). Polyclonal Snk antisera were made to study the protein in the cell, and Fig. 1A shows that the antisera are highly specific for Snk and do not cross-react with either Plk1 or Plk3. To study Snk expression in the cell cycle, NIH 3T3 cells were synchronized by serum starvation and restimulated to enter the cell cycle. Cell lysates were subjected to immunoprecipitation and Western blot analysis with Snk antisera. As shown in Fig. 1B, while Snk was not detected in serum-starved cells, it was present in cells that were stimulated with serum for 1 h. The levels of Snk quickly decreased and were barely detectable at 2 h. In cells that were stimulated for 4, 8, or 25 h, as well as those enriched for S or M phase population by treatment with hydroxyurea or nocodazole, no appreciable level of Snk was detected (Fig. 1B, lanes S and M). Snk expression was also investigated in cells that reentered G1 after mitosis. NIH 3T3 cells arrested at M phase with nocodazole were collected by mechanical shake-off and released into the cell cycle. Western blot analysis revealed that Snk started to appear 140 min after the release from the mitotic arrest, and the highest level was detected at 250 min (Fig. 1C). Only a small amount of Snk was still present 480 min after the release. On the other hand, the mitotic polo-like kinase, Plk, was most abundant in mitotic cells and nearly undetected by 70 min after the release. Cyclin B1, which is destructed at the end of mitosis, was completely degraded by 70 min after the mitotic release. These results indicate that Snk is expressed in early G1 during G1-G0 transition as well as in cycling cells.

The brief expression of Snk in serum-stimulated cells suggested that Snk is turned over quickly. A pulse-chase experiment was carried out to determine the stability of endogenous Snk. Serum-starved NIH 3T3 cells were stimulated with serum in a methionine-free medium supplemented with [35S]methionine. The radiolabel was washed away with normal medium after 30 min, and cells were collected at various time points. Snk was immunoprecipitated from cell lysates using Snk antisera and analyzed by SDS-PAGE and autoradiography. The results showed that nascent Snk is rapidly degraded, with a half-life of about 15 min (Fig. 1D).

**Analysis of the Kinase Activity of Snk**

The kinase activity of endogenous Snk was investigated in immunocomplex kinase assays. Snk was precipitated with Snk antisera from cells that had been stimulated with serum for 1 h, and immunocomplex kinase assay was carried out as described in “Materials and Methods.” The reaction mixtures were resolved by SDS-PAGE and analyzed by autoradiography. Casein was highly phosphorylated when incubated with the immunocomplex prepared from serum-stimulated cells (Fig. 2A). Phosphorylation of casein was not observed when serum-starved cells were used or when Snk antisera were preincubated with the immunogenic peptide (Fig. 2A), suggesting that phosphorylation of casein is due to Snk kinase activity. Phosphoamino acid analysis of phosphorylated casein indicated that only serine and threonine residues were phosphorylated (data not shown).

Because Snk has two highly conserved domains, including a serine/threonine kinase domain at the NH2 terminus and a polo-box at the COOH terminus, point mutations were generated in these domains to study their function in Snk kinase activity. Thr-236, which aligns well with a putative activation phosphorylation site among serine/threonine kinases (38), was substituted with several amino acid residues. The wild-type and mutant Snk proteins were tagged with HA-epitope and...
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The Activity of Snk in Cultured Cells

Among the three mammalian Plks, constitutive expression of Plk has been reported to produce malignant transformation of NIH 3T3 cells, whereas overexpression of Plk3 resulted in incomplete cytokinesis and apoptosis (31, 39). To investigate Snk function in the cell, it was ectopically expressed in COS-7 cells, and confocal immunofluorescence microscopy was used to examine the cells expressing Snk. Cells grown on coverslips were transfected with plasmids encoding Flag-tagged Snk. After 24 h incubation, cells were fixed and those expressing Flag-Snk were detected with monoclonal anti-Flag antibody and labeled with FITC-conjugated secondary antibody. Snk was observed in the cytoplasm of cells. Moreover, cells expressing Snk showed an arborized morphology, with multiple processes extending from the cell body (compare Fig. 3A and B). Some Snk-expressing cells assumed a round morphology (Fig. 3C). Quantitation of the morphology of cells expressing Snk showed that cell arborization appeared to take place before their assuming the round morphology, as a cell population incubated 40 h after transfection showed an increased number of round cells (Table 1). Among the round cells, some had an intact nucleus, whereas a few showed chromosome condensation and appeared to be apoptotic (Fig. 3C).

Expression of Snk mutants further showed that changes in cell morphology are specifically caused by an activity of Snk. The kinase activity is critical, in that the kinase-deficient mutant, Snk.K108M, did not result in any significant changes, whereas Snk.T236E, which has an elevated kinase activity, caused cell arborization and rounding more rapidly (Table 1). However, Snk.fs, despite of having a kinase activity that is comparable to that of the wild-type enzyme, showed a greatly reduced capacity to induce morphological changes (Table 1), suggesting that the polo-box is also required.

A prior study showed that the polo-box of the mitotic Plk is involved in its cellular localization and activity. Here we examined COS-7 cells expressing a GFP-C-Snk, which carries an N-terminal GFP tag that swapped a peptide from Met-1 to Ala-309 in the kinase domain. Using a confocal fluorescence microscope, GFP-C-Snk was shown to localize primarily to an area close to the nucleus in live unfixed cells (Fig. 4A). This area was determined to be the microtubule organizing center (MTOC) when cells were fixed and stained for α-tubulin (Fig. 4D–F). The cellular localization of GFP-C-Snk appeared to be dependent on the microtubule network, in that treating the cells with choline, which depolymerizes microtubules,
resulted in dispersed GFP-C-Snk signal (Fig. 4B). In contrast, the localization of GFP-C-Snk was not altered in cells treated with cytochalasin D, which disrupts the actin network (data not shown). However, GFP-C-Snk.fs, which carries the frameshift mutation in the polo-box, showed a rather dispersed localization in the cytoplasm (Fig. 4C), suggesting that the polo-box is involved in cellular localization of Snk.

Functional Interaction of Snk With the Calcium- and Integrin-Binding Protein CIB

Our previous studies with yeast two-hybrid screening identified a Snk-interacting protein, which was deposited into the Genbank in 1996 (accession no. U83236). This protein was independently identified as the calcium- and integrin-binding protein (CIB) (40). Kauselmann et al. (37) subsequently reported the Snk and CIB interaction using yeast two-hybrid system and colocalization studies. Here we sought to test whether CIB has any effect on Snk activity in the cell. Plasmids encoding Flag-Snk and HA-CIB were co-transfected into COS-7 cells, and cell morphology was examined by immunofluorescence confocal microscopy. Cells expressing Flag-Snk were stained with FITC-conjugated antibody, while cells expressing HA-CIB were stained with Cy5-conjugated antibody. The results showed that HA-CIB was detected both in the nucleus and cytoplasm and that the expression of HA-CIB did not cause any apparent changes in cell morphology (Fig. 5, indicated by an asterisk). That CIB was able to prevent Snk-induced morphological changes was further supported by scoring cells with various morphologies (Table 1). CIB also appeared effective in inhibiting the capacity of the constitutively active mutant, Snk.T236E, to induce morphological changes (Table 1).

To investigate whether CIB and Snk interacted in COS-7 cells, a co-immunoprecipitation assay was carried out. Plasmids encoding HA-CIB and Flag-Snk were co-transfected into COS-7 cells, and detergent-soluble cell lysates were prepared after 27 h incubation. The lysates were subjected to immunoprecipitation using monoclonal antibody specific for HA or Flag epitope, and the presence of Flag-Snk and HA-CIB in the immunocomplexes was detected by Western blot analysis with anti-Flag and anti-HA antibody, respectively. Western blot analysis showed that HA-CIB was present in the immunocomplex precipitated by anti-Flag antibody, and Flag-Snk was present in the anti-HA immunocomplex (Fig. 6A). The results suggest an association of the two proteins in COS-7 cells. One

![FIGURE 4.](image)

**FIGURE 4.** Cellular localization of GFP-C-Snk. A–C. Images taken from live unfixed COS-7 cells expressing GFP-C-Snk (A and B) or GFP-C-Snk.fs (C), which carries a frameshift mutation disrupting the polo-box. Cells were treated with colchicine in B, D–F. Images of cells that were fixed and stained for α-tubulin. E. Cell expressing GFP-C-Snk. E. Microtubule network. F. Merged image of D and E, showing the localization of GFP-C-Snk to the microtubule organization center. Scale bar, 25 μm.
caveat is that CIB was reported to contain a myristoylation site at the NH2 terminus (41), which could be disrupted in HA-CIB. Myristoylation of CIB and its subsequent localization to the membrane may have an effect on its interaction with Snk in the cell.

There are at least two scenarios whereby an association of CIB can affect Snk activity in the cell. It is possible that CIB may interfere with the function of the polo-box, or CIB may affect the kinase activity of Snk. To differentiate these two possibilities, immunocomplex kinase assays were carried out to determine whether the interaction with CIB has any effect on Snk kinase activity. Plasmids encoding Flag-Snk and HA-CIB were co-transfected into COS-7 cells. After incubation for 24 h, Flag-Snk was immunoprecipitated from cell lysates and assayed for its kinase activity. As shown in Fig. 6B, CIB diminished autophosphorylation of Snk and its phosphorylation of casein. The results suggest that CIB is an inhibitor of Snk kinase activity.

**Discussion**

The serum-inducible kinase Snk was identified as an immediate-early transcript in NIH 3T3 cells, and its deduced amino acid sequence suggested that it is a member of the Plks (29). In this study, the expression of Snk in the cell cycle was examined. Endogenous Snk was detected in serum-starved NIH 3T3 cells that were stimulated with serum for 1 h. The amount of Snk rapidly decreased and became undetectable 4 h after stimulation. These observations are consistent with the expression profile of Snk message that was reported previously and suggest that Snk is expressed in G1 (29). In cells that were blocked at S or M phase, Snk was not detected. To examine whether Snk is expressed in cycling cells that reenter G1, NIH 3T3 cells were treated with nocodazole and subsequently released from the resultant mitotic arrest. Snk appeared 70 min after the complete degradation of cyclin B1, and because destruction of cyclin B1 occurs at the end of mitosis (42), the data suggest that Snk is expressed in early G1. The mitotic release experiment also showed that Plk is degraded before the expression of Snk. In a similar experiment, the third member of the murine Plks, Fnk, was shown to have altered electrophoretic mobility when cells exit mitosis although its amount remained constant (23). An apparent overlapping of the expression of Fnk with that of Plk and Snk raises a possibility of functional redundancy among these Plks. Plk and Prk (Fnk) were both shown to complement mitotic defects caused by CDC5 mutation in budding yeast (30, 43); however, our unpublished results show that Snk fails to efficiently complement the cdc5-1 temperature-sensitive defect in Saccharomyces cerevisiae (data not shown). This absence of detectable Snk expression and function in M phase strongly indicates that Snk is a member of the non-M-phase Plks.

In addition to the cell cycle regulation of Snk expression, endogenous Snk appears to be subjected to post-transcriptional controls. Using pulse-chase analysis, nascent Snk was shown to have a half-life of about 15 min. The mitotic Plks such as Cdc5 and Plk are substrates for the APC (12, 15, 44). Interestingly, a sequence of Snk from Arg-180 to Ser-188 is similar to that of the destruction box of cyclins (45), and a potential KEN box for Cdh1-APC is found in Snk from Lys-149 to Asn-151 (46). Because the KEN box is shown to regulate proteolysis in G1 (47), it is possible that Snk is degraded through this pathway. Moreover, a PEST sequence, which is present in many unstable proteins, is identified from Arg-412 to Arg-426 in Snk using the PEST-FIND computer program (48). It appears that as is the case for mitotic Plks, regulation of Snk levels may be an important mechanism for modulating its activity in the cell.

The kinase activity of endogenous Snk was demonstrated by phosphorylation of casein with immunocomplex kinase assays. As expected, Snk phosphorylated serine and threonine residues (data not shown). Studies of murine Plk and *Xenopus* Plx1...
showed that an acidic substitution of a threonine residue in a putative activation loop of the kinase domain resulted in elevated kinase activity (3, 43). A similar substitution in Snk, where Thr-236 was changed to aspartate, caused about 10-fold increase in Snk kinase activity. Changing Lys-108 in the putative ATP-binding domain to a methionine abolished the kinase activity of Snk. It was reported previously that mutation in the polo-box did not affect Plk kinase activity (49), and this appears to be the case in Snk as well. Disruption of the most conserved region in the polo-box by a frameshift mutation showed no significant effect on the kinase activity of Snk. These studies with Plk and Snk suggest that conserved mechanisms may be involved in regulating the activity of M and G1 Plks.

Functional analysis of Plk in previous studies suggested that both the kinase activity and the polo-box are required for in vivo mitotic functions of the enzyme (49). Lee et al. (49) showed that the polo-box is involved in cellular localization of Plk. In this report, we showed that both the kinase activity and the polo-box are required for Snk activity in the cell and that the polo-box is involved in cellular localization of Snk. COS-7 cells expressing Snk showed an arborized cell morphology, with extended processes growing out of the cell body. Expression of the kinase-deficient mutant of Snk did not cause any significant morphological changes, nor did the expression of the polo-box frameshift mutant of Snk, although this mutant has a kinase activity comparable to that of the wild type. On the other hand, the mutant with an elevated kinase activity produced changes more rapidly. These data suggest that perhaps Snk contributes to morphological changes in the cell as it exits mitosis. It is interesting that the expression of Snk in cells reentering G1 after mitotic arrest coincided with the flattening and spreading of round mitotic cells, which was clearly visible under a light microscope (data not shown). It is possible that Snk may be involved in the reorganization of the cytoskeleton after mitosis. Support for this is the localization of an NH2-terminally tagged GFP-C-Snk fusion protein to the MTOC in a polo-box-dependent fashion. Recently, Plk3 has been reported to localize to the centrosome (50), and Sak, which shares homology with plks in the COOH terminus but lacks a clearly defined polo-box, also showed localization to the centrosome (51). Thus, the presence of the polo-box and unidentified sequence elements appears to predict subcellular localization of plks. It is noteworthy that in contrast to our results, when a Snk-GFP fusion protein was used to study cellular localization of Snk in COS-7 cells, no morphological changes were reported, and the fusion protein did not show specific localization to MTOC (37). It is possible that the GFP, which in this case was fused to the COOH terminus of Snk, may have disrupted the structure of the COOH terminus of Snk and the function of the polo-box.

COS-7 cells expressing Snk for an extended time showed signs of apoptosis such as chromosome condensation. Expression of a hyperactive mutant of Snk, Snk.T236E, caused more cells to appear apoptotic 24 h after transfection. Given that endogenous Snk is present at low levels and quickly turned over in vivo, it appears that unregulated Snk activity is detrimental to the cell. Therefore, the apoptosis-like phenotype induced by Snk may be an artifact of unregulated expression of Snk. Others have reported that ectopic expression of Plk3 induced apoptosis (31), and it is possible that the apparent apoptosis in cells expressing Snk is caused by phosphorylation of substrates that Snk and Plk3 have in common.

We originally identified the calcium- and integrin-binding protein CIB as a Snk-interacting protein (Genbank accession no. U83236, 1996) using a yeast two-hybrid screening. Subsequently, Kauselmann et al. (37) reported on the interaction of Snk and CIB and demonstrated their colocalization in neuronal cells. In this study, we tested whether CIB has any effect on Snk activity in the cell. Co-transfection of CIB with Snk diminished the capacity of Snk to induce morphological changes. Because CIB and Snk co-immunoprecipitated from the cell lysate and that an immunocomplex kinase assay revealed that co-expression of CIB with Snk decreased Snk kinase activity, it appears that the association of CIB with Snk may inhibit the kinase activity of Snk. Calmodulin, which shares sequence homologies with CIB, modulates activity of CaM-kinas through binding to the non-catalytic domain (51). It is also possible that CIB may inhibit Snk kinase activity by recruiting a phosphatase, similar to the action of calcineurin B (40), although this appears unlikely, as CIB apparently inhibited the activity of a constitutively active mutant, Snk.T236E (Table 1). Thus, CIB seems to be a direct modulator of Snk kinase activity. Yeast two-hybrid interaction analysis showed that CIB interacted strongly with the full-length Snk, whereas its interaction with the NH2-terminal kinase domain or the COOH-terminal domain, which retained an intact polo-box and other conserved sequences, was negligible (data not shown). Thus, it appears that CIB recognizes a structural feature in full-length Snk. The mechanism of the interaction of CIB with Snk merits further investigation, as CIB also showed interaction with Fnk (32) and could provide a phosphorylation-independent mechanism for the regulation of the kinase activity of Plks. It should also be considered that CIB contains a myristoylation sequence and can be myristoylated and localize to the cell membrane (41), which may affect its interaction with Snk and Fnk.

Co-expression experiment showed that the inhibitory effect of CIB on Snk-induced morphological changes diminished over time, as cells co-transfected with CIB and Snk showed an increase in abnormal morphologies after incubation for 40 h. This may be caused by changes in relative amount of Snk and CIB in the cell, or it is possible that the interaction between CIB and Snk is affected by other cellular factors. For example, CIB has two EF-hand motifs and binds Ca2+ (40), and previous studies have shown that Ca2+ is able to mediate the interaction of CIB with integrin (52). Therefore, it is possible that the interaction of CIB with Snk is affected by cellular Ca2+ concentration. This may be relevant to the role of Snk in neurons, where CIB and Snk showed colocalization and have the potential to regulate neuronal activities (37).

**Materials and Methods**

**Cell Culture and Chemicals**

COS-7 and NIH 3T3 cells were obtained from American Type Culture Collection and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Intergen, Purchase, NY) or 10% heat-inactivated normal calf serum.
(Intergen), respectively. Penicillin G (100 units/ml) and streptomycin (100 μg/ml) (Life Technologies, Inc., Rockville, MD) were included in the medium. Cells were incubated at 37°C in 8% CO₂. Serum starvation of NIH 3T3 cells was carried out by incubation in DMEM plus 0.5% calf serum for 30 h, and cells were stimulated with 10% calf serum. NIH 3T3 cells were treated with 1 mM hydroxyurea or 200 ng/ml of nocodazole for 18 h to obtain S or M phase-enriched cell population. To study Snk expression in cells that reenter the cell cycle, NIH 3T3 cells were treated with 200 ng/ml of nocodazole for 18 h and collected by mechanical shake-off. These cells were washed three times with cold DMEM plus 10% calf serum and then incubated in pre-warmed growth medium. Colchicine treatment of COS-7 cells was carried out for 2.5 h at 37°C at a final concentration of 40 μg/ml. Transient transfection of COS-7 cells was performed using the Calcium Phosphate Transfection Kit (5 Prime-3 Prime Inc., Boulder, CO) or the GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA), following the manufacturer’s protocols. Chemicals were purchased from Sigma, St. Louis, MO unless stated otherwise.

Expression of Snk in Insect Cells and Mutagenesis of Snk

HA-Snk was produced by fusing an NH₂-terminal HA epitope to the second amino acid residue of Snk by cloning a SacI-EcoRI fragment of Clone 2 (29) into a baculovirus transfer vector, pAc702 (Invitrogen), and expressed in SF9 cells using the BaculoGold Baculovirus Expression Vector System (PharMingen, San Diego, CA). Point mutations were generated in Snk by site-directed mutagenesis using a kit purchased from Amersham, Piscataway, NJ. Mutations were confirmed by DNA sequencing. A frameshift mutation disrupting the polo-box was generated by cutting pSM227 at BstEII site just 5’ to the polo-box, followed by blunt-end ligation after S1 nuclease treatment. The COOH-terminal amino acid sequence was brought back into its original frame by cutting the resultant plasmid at a KpnI site in the polo-box, treating with S1 nuclease, and religation.

Immunoprecipitation, Western Blot Analysis, and Immunocomplex Kinase Assays

Antisera were raised against an NH₂-terminal peptide of Snk, corresponding to amino acid residues Asp-29 to Gly-42, and the COOH terminus, from Ser-310 to Asn-682, that was purified from insect cells as a GST fusion protein. The affinity-purified antisera were able to detect ectopically expressed Snk and various truncated Snk fragments with expected electrophoretic mobility (data not shown). To detect endogenous Snk from NIH 3T3 cells, cells were lysed in 1 ml of radio-immunoprecipitation assay (RIPA) buffer [10 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% sodium deoxycholic acid, 1% Triton X-100, 0.1% SDS], and the lysates were clarified by centrifugation at 100,000 x g for 30 min. Snk antisera (3–4 μg) were added to the supernatant and incubated for 1 h at 4°C. Protein A-conjugated with Sepharose 4B (Zymed, San Francisco, CA) was added and incubated for 30 min. Precipitated immunocomplexes were washed three times with RIPA buffer, resolved by SDS-PAGE, and subjected to Western blot analysis using Snk antisera following protocols published previously (53). For immunocomplex kinase assays, RIPA buffer was substituted with Lysis buffer A [10 mM potassium phosphate (pH 7.1), 1 mM EDTA, 5 mM EGTA, 10 mM MgCl₂, 2 mM DTT, 2 mM Na₃VO₄, 50 mM β-glycerophosphate, 0.5% Triton X-100]. Anti-HA (12CA5, 2.8 μg) or anti-Flag (M2, 5 μg) antibodies were used to precipitate HA-Snk from SF9 and Flag-Snk from COS-7 cells, respectively. The immunocomplexes were washed twice with Lysis buffer A, once with ST [50 mM Tris-HCl (pH 7.2), 150 mM NaCl] containing 1 mM DTT, and once with kinase buffer [25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT]. Kinase assays were performed by incubating the immunocomplexes in 30 μl of kinase buffer containing 5 μCi of [γ-32P]ATP, 166 μg/ml myelin basic protein (MBP) or casein, 0.1 mg/ml ovalbumin, and 50 μM ATP for 30 min at 30°C.

Metabolic Labeling of Snk in NIH 3T3 Cells

To determine the half-life of endogenous Snk, pulse-chase experiments were performed. Serum-starved NIH 3T3 cells were washed with serum- and methionine-free DMEM medium (Life Technologies) and incubated in this medium for 30 min. Trans-35S label (ICN) and bovine calf serum were then added to a final concentration of 100 μCi/ml and 10%, respectively. Cells were grown at 37°C for 30 min before the radiolabel was washed off with DMEM plus 10% serum. Cells were further incubated in DMEM containing 10% serum for the time indicated in the text. Cell lysates were prepared and subjected to immunoprecipitation with Snk antisera. The immunocomplexes were resolved by SDS-PAGE, and 35S-labeled Snk was detected by autoradiography.

Ectopic Expression of Snk and Indirect Immunofluorescence Microscopy

To ectopically express Snk in COS-7 cells, a Flag-epitope was fused to the Snk polypeptide that is encoded by a SacI-EcoRI fragment and deleted of the first methionine of Snk. The resultant plasmid, pSM227, expresses the fusion protein from a cytomegalovirus (CMV) immediate-early promoter. The kinase-deficient (Snk.K108M) and constitutively active (Snk.T236E) mutants were similarly expressed. For indirect immunofluorescence studies, COS-7 cells were seeded on 22 x 22 mm acid-washed glass coverslips in a six-well tissue culture plate and grown for 16–20 h. Cells were transfected with plasmid DNA and further incubated for the time indicated in the text. After two washes with PBS, cells were fixed at 4°C for 30 min in 4% paraformaldehyde-PBS (pH 7.4). Fixed cells were washed three times with PBS and permeabilized with cold methanol for 2 min. The coverslips were then blocked with 6% BSA for 30 min at room temperature. Primary antibodies were applied overnight at 4°C and secondary antibodies were applied for 30 min at 25°C. Monoclonal anti-Flag M2, anti-HA 12CA1, and rabbit polyclonal anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted to a final concentration of 5 μg/ml in PBS containing 3% BSA; FITC-conjugated anti-mouse IgG (Boehringer Mannheim, Mannheim, Germany) and Texas Red-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR) were diluted 1:200 in PBS containing 1% BSA. Propidium
iodide was applied at a concentration of 40 μg/ml for 30 min at 25°C to stain nuclear DNA. After three washes with PBS, coverslips were mounted in PBS containing 20% glycerol and 25% 1,4-diazabicyclo[2.2.2]-octane (Aldrich, Milwaukee, WI). Immunofluorescence microscopy was performed with a Zeiss Axiosvert 100 TV confocal fluorescence microscope, and recorded images were printed with Adobe Photoshop software. To score cells with various morphologies, slides were counted by two individuals—two of whom had no prior knowledge of the experiments.

To study cellular localization of Snk, an NH2-terminal NheI-NheI fragment in pSM227 was substituted with a XbaI-XbaI fragment encoding the green fluorescence protein (GFP), resulting in pSM242. As described above, GFP-C-Snk was expressed in COS-7 cells, and cells were examined with a confocal fluorescence microscope. To observe GFP-C-Snk localization in live cells, cells were mounted in PBS, and pictures were taken within 5 min. To stain for the microtubule network, cells were fixed and permeabilized in microtubule-stabilization buffer (54) and then applied with monoclonal anti-α-tubulin (1:1000) and Texas Red-conjugated anti-mouse IgG (Molecular Probes, 1:200).

Interaction of CIB and Snk

An HA-tag was added at the NH2-terminal end of CIB, and HA-CIB was expressed using the elongation factor promoter from pEF-BOs-VC, a gift from Anjana Rao. For co-immunoprecipitation assays, plasmids encoding HA-CIB (4 μg) and Flag-Snk (16 μg) were co-transfected into COS-7 cells and incubated at 37°C for 24 h. Cells were treated with 40 μg/ml of colchicine for 3 h before harvesting. Immunoprecipitation of HA-CIB and detection of Flag-Snk in the immunocomplexes by Western blot analysis were carried out as described above, except for the following modifications. Cells were lysed in lysis buffer [1% Triton X-100, 50 mM NaCl, 50 mM Tris-Cl (pH 7.7 at 4°C), 1 mM EDTA, 100 μg/ml leupeptin, 50 μM β-mercaptoethanol] containing 0.25% Triton X-100. The immunocomplexes were washed twice with IP buffer and then resolved by SDS-PAGE. Immunoprecipitation of Flag-Snk and detection of HA-CIB in the immunocomplexes were performed using lysates of cells transfected with plasmids encoding Flag-Snk (4 μg) and HA-CIB (16 μg).

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