

STATHMIN BINDS HEAT-SHOCK-PROTEIN 70

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ABSTRACT. Stathmin is a cytosolic protein postulated to act as an intracellular relay in cell signaling. It interacts via coiled-coil motifs with several proteins, including BiP/Grp78 and Hsc70, two members of the heat-shock-70 (Hsp70) family of proteins. In the current study, a glutathione-S-transferase (GST)-stathmin fusion protein was used to determine whether stathmin also binds to Hsp70, a third member of the Hsp70 family. Stathmin-binding proteins were examined in Nb2 cells, a rat lymphoma, and in PC12 cells, a rat pheochromocytoma. Prolactin (PRL)-stimulated Nb2 cells were used as the primary source of cytosol and membrane proteins because it has been demonstrated that PRL induces expression of Hsp70 in these cells. PC12 cells were used because they are of neuronal origin, and stathmin appears to play important roles in neural signaling. The results showed that stathmin binds multiple Nb2- and PC12-cell proteins. Immunostaining confirmed that stathmin binds to tubulin and showed for the first time that stathmin binds to Hsp70.

Keywords: Stathmin, Nb2, PC12, Hsp70, glutathione-S-transferase

Stathmin is a ubiquitous, cytosolic phosphoprotein believed to be involved in intracellular signaling (Sobel 1991). Though its signaling mechanism is yet unclear, stathmin is known to associate with several proteins via coiled-coil interactions (Larsson et al. 1999; Li & Cohen 1996; Maucuer et al. 1995; Redeker et al. 2000). Proteins found to interact with stathmin include tubulin (Belmont & Mitchison 1996; Curmi et al. 1997; Gradin et al. 1998; Horwitz et al. 1997; Jourdain et al. 1997; Larsson et al. 1999; Redeker et al. 2000), the tumor susceptibility protein TSG101 (Li & Cohen 1996; Maucuer et al. 1995), the serine/threonine kinase KIS (Maucuer et al. 1995), and members of the heat-shock-70 (Hsp70) family of proteins (Manceau et al. 1999; Maucuer et al. 1995). The two members of the Hsp 70 family shown to bind to stathmin are BiP/GRP 78 (Maucuer et al. 1995) and Hsc70 (Manceau et al. 1999). Evidence suggests that Hsc70 preferentially binds to unphosphorylated stathmin (Manceau et al. 1999), as does tubulin (Curmi et al. 1997; Gradin et al. 1998; Horwitz et al. 1997; Jourdain et al. 1997).

Interaction of unphosphorylated stathmin with tubulin appears to increase the catastrophe rate of microtubules (Belmont & Mitchison 1996). Stathmin may promote shortening of microtubules by sequestering tubulin or by directly interfering with microtubule polymer-

ization. The importance of the interaction of stathmin with members of the Hsp family is less well understood. However, interaction of stathmin with members of the Hsp70 family opens additional pathways for regulation of cell growth and development by stathmin.

Given the known interactions with other members of the Hsp70 family, we attempted to determine whether Hsp70 itself interacts with stathmin using a probe consisting of stathmin coupled to glutathione-S-transferase (GST). The probe also was used to identify stathmin-binding proteins in the Nb2 lymphoma and PC12 cells.

METHODS

Ovine prolactin (oPRL; NIH P-1-2) was obtained from the Hormone Distribution Program, NIH (Bethesda, Maryland). Anti-Hsp70 (K-20), which does not cross-react with Hsc70, was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California). Anti-tubulin and anti-glutathione-S-transferase were obtained from Sigma Chemical Company (Saint Louis, Missouri).

GST-stathmin fusion protein.—A cDNA coding for the entire human stathmin gene was cloned into a SmaI/SalI site in the pGEX-5X-1 GST fusion vector (Amersham Pharmacia Biotech, Piscataway, New Jersey). The 5' end of the construct was sequenced to ensure that the cDNA was cloned into the cor-

rect frame. The vector was transformed into competent *E. coli* TG1 cells and expression of the GST-stathmin fusion protein was induced by addition of isopropyl- β -D-thiogalactoside (IPTG, 100 mM). The stathmin-GST fusion protein was extracted from the TG1 cells according to the manufacturer's recommendations (GST Gene Fusion System, Amersham Pharmacia Biotech), recovered on glutathione-Sepharose, washed, and eluted using glutathione (10 mM). GST was obtained by expression from pGEX-5X-1 not containing an insert. Protein recovered from glutathione-Sepharose was further purified by FPLC on Superdex 75 (Amersham Pharmacia Biotech). Column fractions containing GST-stathmin or GST were collected, re-purified on glutathione-Sepharose, and washed and concentrated in a Centricon 10 (Amicon Inc., Beverly, Massachusetts).

³⁵S-labeling of Nb2 and PC12 proteins.—Nb2 cells, a rat lymphoma, were grown in Fischer's Medium (for leukemic mouse cells) containing 10% horse serum, 10% fetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol, 100 units of penicillin/ml, and 100 μ g of streptomycin/ml (Meyer et al. 1992). Proliferating Nb2 cells (approximately 2×10^7) were washed and resuspended in 3 ml of a methionine- and cysteine-free medium (Minimum Essential Medium Eagle without methionine, cysteine, Sigma Chemical Company) supplemented with prolactin (PRL, 10 ng/ml), 0.1 mM 2-mercaptoethanol, 10% horse serum and a mixture containing ³⁵S-methionine and ³⁵S-cysteine (1 mCi, Express³⁵S³⁵S, NEN Life Science Products, Boston, Massachusetts). PRL stimulates growth of Nb2 cells in the absence of FBS; thus, FBS could be eliminated from the labeling medium. Reduction of serum promotes stronger labeling of proteins with the ³⁵S-methionine/³⁵S-cysteine mixture, because FBS contains significant amounts of unlabeled cysteine and methionine. PC12 cells were grown in RPMI 1640 supplemented with 10% horse serum, 5% FBS, 100 units of penicillin/ml, and 100 μ g of streptomycin/ml. Proliferating PC12 cells (approximately 1×10^7) were suspended in 3 ml of the methionine- and cysteine-free medium supplemented with 10% horse serum, 5% FBS, and 1 mCi of the ³⁵S-methionine/³⁵S-cysteine mixture. FBS was added to the labeling incubation to maintain the viability of the PC12 cells. All

cells were incubated in their respective media for 6 h at 37° C in CO₂/air (1:19).

Cells were collected by centrifugation at $400 \times g$ for 4 min at 4° C, washed twice in Tris-saline buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0), and resuspended in Tris-saline containing proteinase inhibitors [pepstatin (10 μ g/ml), leupeptin (10 μ g/ml), and aprotinin (0.2 TIU/ml)]. Nb2 cells were passed 10 times through a 23-gauge needle and centrifuged at $12,000 \times g$ for 1 min at 4° C. The supernatant fraction (cytosol) was removed from the pellet, passed through a 0.45 μ m filter, and washed and concentrated in a Centricon 10 (Amicon, Inc.). The crude membrane pellet was suspended in Tris-saline containing 1% Triton X-100, 2mM EDTA and proteinase inhibitors, passed 10 times through a 23-gauge needle, and centrifuged at $12,000 \times g$. The supernatant fraction containing solubilized membranes was removed for use in binding studies.

Because many of the PC12 cells adhere to the culture dish, they were solubilized at 4° C in Tris-saline buffer containing 1% Triton X-100 and proteinase inhibitors. The solubilized cells were centrifuged at $12,000 \times g$ for 1 min and the supernatant fraction was passed through a 0.45 μ m filter. PC12 proteins were washed and concentrated in a Centricon 10.

Binding.—Proteins extracted from PC12 cells (whole cell) and from Nb2 cells (cytosol or crude membrane) were incubated with 20 nanomoles of the GST-stathmin fusion protein or equivalent amount of GST protein for approximately 18 h at 25° C. Incubations were performed in a Tris-saline buffer (50 mM Tris-HCl, 150 mM NaCl) containing the proteinase inhibitors. The pH of the buffer was adjusted to 8.0 or 6.8 as noted in figure legends, Triton X-100 was maintained at 0.05–0.1% in solutions containing solubilized membranes, and MgCl₂ was added to a final concentration of 5 mM where noted. After incubation, 25 μ l of packed glutathione-Sepharose 4B was added to each reaction and the tubes were shaken gently for 30 min at 25° C. The amount of glutathione on the Sepharose was sufficient to bind all the GST (free or associated with the stathmin-GST fusion protein) in the reaction. Therefore, the beads rapidly precipitated the stathmin-GST fusion protein and proteins associated with the fusion protein. The Sepharose beads were collected by centrifugation,

washed 3 times in Tris-saline buffer, and stathmin-GST fusion protein were separated from the Sepharose (solubilized) using 10 mM glutathione.

Electrophoresis and immunostaining.—In each experiment, equal volumes of the glutathione eluates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) on 10% polyacrylamide gels. Electrophoresis was run on a Mini-Protein II vertical gel system at a constant voltage of 125 V for approximately 90 min, at which time the dye front reached the bottom of the gel. Proteins in the gels were electrophoretically transferred (60 volts, 1 h) to nitrocellulose paper (0.22 μ m) using a Trans-Blot Cell (Bio-Rad Laboratories, Hercules, California) and the buffer system described by Towbin et al. (1979). Open sites on the nitrocellulose paper were blocked by incubation in binding buffer (100 mM Tris-HCl, 0.15 M NaCl, 0.1% BSA, pH 8.0) containing 5% non-fat dry milk. After three 10-min washes in binding buffer, the nitrocellulose was incubated for 18–20 h in binding buffer containing the primary antibody. Unbound antibody was removed by three 10-min washes in binding buffer, and the nitrocellulose was incubated for 1 h with a secondary antibody coupled to alkaline phosphatase. Unbound antibody was removed by three 10-min washes in binding buffer, and the alkaline phosphatase was detected using OneStep BCIP/NBT (Pierce Chemical Co., Rockford, Illinois).

RESULTS

The GST-stathmin fusion protein and GST expressed at high levels in the gene fusion system and provided relatively pure probes for analyzing stathmin-binding proteins.

The GST-stathmin fusion protein bound several Nb2-cell proteins. Figure 1 shows 35 S-labeled proteins isolated on glutathione-Sepharose from cytosol and crude-membrane extracts after addition of no probe (C), GST (T) or GST-stathmin (S) at pH 8.0. Several cytosolic proteins (noted by asterisks on figure) in the 40–92-kDa range were more prominent in the GST-stathmin lane, and thus may represent protein bound to stathmin directly or via another stathmin-binding protein. A prominent 50 kDa-protein preferentially bound by GST-stathmin appeared to be present in both

the cytosol and membrane fractions. A protein(s) migrating at approximately 70 kDa appeared to be represented in both cytosol and membrane fractions, but was more prominent in the membrane fraction.

It has been shown (Curmi et al. 1997) that the stathmin-tubulin interaction is stronger at a pH below 7.0 and in the presence of MgCl_2 . Therefore, binding of stathmin to 35 S-labeled proteins extracted from Nb2 cells was examined at pH 6.8 in the presence of 5 mM MgCl_2 (Fig. 2). The pattern of proteins bound by the GST-stathmin probe clearly differed from that obtained at pH 8.0. Several proteins in the 25–69-kDa range (noted by asterisks on figure) were present at pH 6.8. A strong protein doublet migrating at approximately 60 kDa was recognized by the GST-stathmin probe in both the cytosol and membrane fractions at pH 6.8. There was no comparable doublet at pH 8.0, though a protein of approximately 60 kDa was identified in experiments performed at pH 8.0.

The GST-stathmin probe also recognized multiple proteins in the 46–92-kDa range (noted by asterisks on figure) in PC12 cells (Fig. 3). A couple of the proteins migrated with apparent molecular weights similar to those of proteins identified in Nb2 cells, but it is unknown whether the proteins identified in the two cells are related. In PC12 cells, a strong protein doublet migrating with an apparent molecular weight of less than 30 kDa was present in all lanes. This doublet was recognized by anti-transferase antibody (data not shown) suggesting that the proteins represent endogenous GST that binds directly to the glutathione-Sepharose beads.

To determine whether stathmin binds Hsp70, 35 S-labeled proteins extracted from Nb2 cells at pH 8.0 were probed with anti-Hsp70 antiserum. The antiserum, which recognizes Hsp70 but not Hsc70, identified a protein at 70-kDa (noted by asterisk on figure) among the proteins recognized by the GST-stathmin fusion protein (Fig. 4A). The immunostained protein appeared to match a 35 S-labeled protein (noted by asterisk on figure) isolated using the GST-stathmin probe (Fig. 4B). Thus, the data suggest that stathmin associates with Hsp70 in Nb2 cells.

Stathmin also appeared to bind tubulin-like proteins from Nb2 cells. When binding was performed at pH 6.8, the GST-stathmin fraction contained two proteins recognized by an

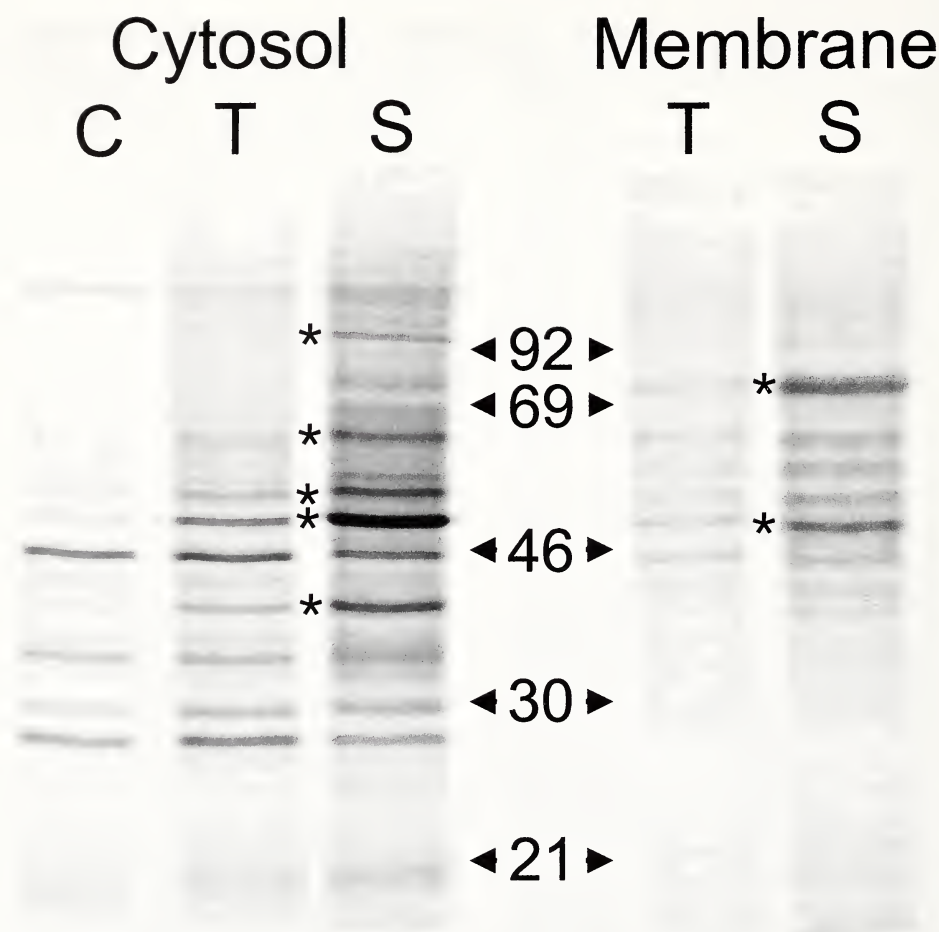


Figure 1.— ^{35}S -labeled proteins in cytosol and membrane fractions from Nb2 cells incubated at pH 8.0 with no additions (C), GST (T) or GST-stathmin (S). Proteins were isolated on glutathione-Sepharose and analyzed by SDS-PAGE and autoradiography. Numbers in center show the positions of molecular-weight standards. Asterisks indicate bands that are more prominent in the GST-stathmin lane.

anti-tubulin antiserum (Fig. 5). The two proteins migrated with apparent molecular weights of 55 kDa (Fig. 5), which are consistent with the molecular weights of α and β tubulin. The 55-kDa proteins were not present in control lanes. Tubulin-like proteins also were not identified when binding was performed at pH 8.0 instead of 6.8, consistent with earlier results (Curmi et al. 1997) showing that stathmin binds tubulin better at the lower pH.

DISCUSSION

Stathmin is believed to interact with several cellular proteins, including two member of the Hsp70 family (Manceau et al. 1999; Maucuer

et al. 1995). Although only two Hsp70-related proteins, BiP/Grp78 (Maucuer et al. 1995) and Hsc70 (Manceau et al. 1999), have been identified as stathmin-binding proteins, it seems possible that other members of the Hsp70 family may also associate with stathmin. Moreover, in view of its postulated role as an intracellular relay protein (Sobel 1991) and its broad distribution, stathmin likely associates with additional proteins. In the current study we have examined whether stathmin binds to additional proteins, including additional members of the Hsp70 family.

Stathmin appears to bind Hsp70. The stathmin-GST fusion protein isolated a protein that reacts with an antibody specific for Hsp70.

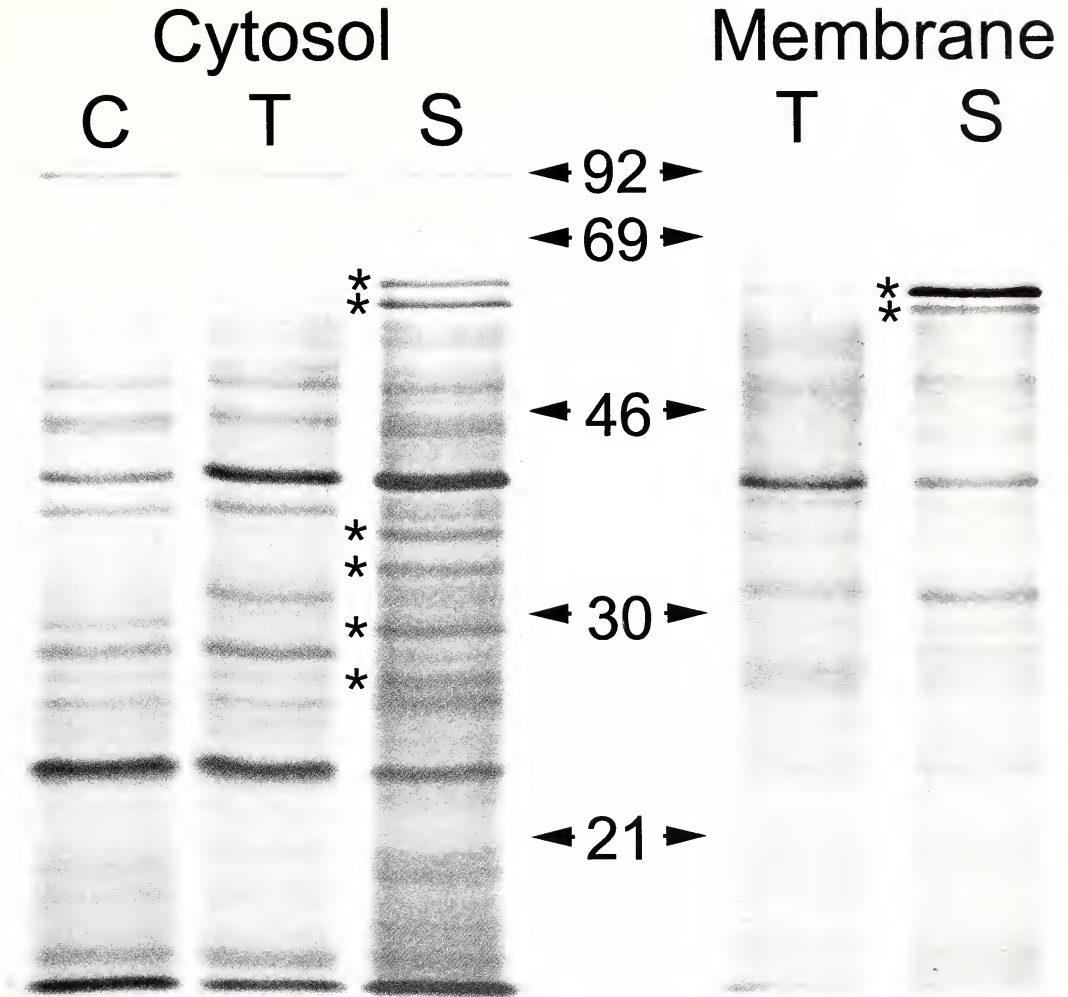


Figure 2.—³⁵S-labeled proteins in cytosol and membrane fractions from Nb2 cells incubated at pH 6.8 and 5 mM MgCl₂ with no additions (C), GST (T) or GST-stathmin (S). Proteins were isolated on glutathione-Sepharose and analyzed by SDS-PAGE and autoradiography. Numbers in center show the positions of molecular-weight standards. Asterisks indicate bands that are more prominent in the GST-stathmin lane.

The immunostained protein is unlikely to be Hsc70 because studies by the vendor indicate antibody used in the study does not cross-react with Hsc70. In Nb2 cells, expression of Hsp70 is induced by PRL and other mitogens (de Toledo et al. 1987; Horn et al. 1994); thus, Hsp70 levels would be expected to be elevated in the asynchronous, PRL-treated cells used in the studies. Induction of Hsp70 appears to be closely associated with Nb2-cell proliferation, but the exact role of Hsp70 is unclear. It is interesting that PRL and phorbol esters induce Hsp70 and stimulate phosphorylation of stathmin in Nb2 cells because Man-

ceau et al. (1999) have suggested that phosphorylated forms of stathmin have reduced affinity for Hsp70-like proteins. Perhaps stathmin sequesters Hsp70, and stathmin phosphorylation releases Hsp70 so it can participate in mitogenesis or the response to heat shock. This type of regulation would be consistent with the stathmin-tubulin interaction where phosphorylation of stathmin decreases its ability to sequester tubulin (Curmi et al. 1997; Gradin et al. 1998; Horwitz et al. 1997; Jourdain et al. 1997). If unphosphorylated stathmin does sequester Hsp70, then phosphorylation of stathmin might be an important

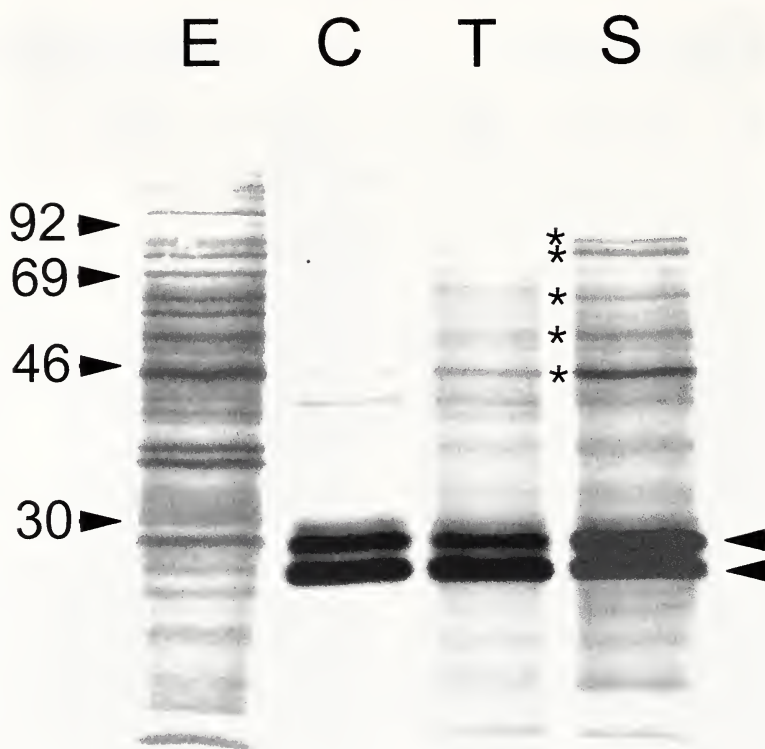


Figure 3.— ^{35}S -labeled proteins in whole-cell lysates of PC12 cells (E) were incubated at pH 8.0 with no additions (C), GST (T) or GST-stathmin (S). Proteins in C, T and S were isolated on glutathione-Sepharose and analyzed by SDS-PAGE and autoradiography. Arrowheads at right indicated the positions of proteins recognized by anti-GST antibodies. Numbers at left show the positions of molecular-weight standards. Asterisks indicate bands that are more prominent in the GST-stathmin lane.

consequence of heat shock as has been shown in HeLa cells (Beretta et al. 1995). In any case, these data suggest, for the first time, that stathmin binds Hsp70. It is possible, therefore, that stathmin-Hsp70 interaction participates in signaling in Nb2 cells.

Stathmin also associated with ^{35}S -labeled proteins with molecular weights (55 kDa) approximating those of α and β tubulin. A ^{35}S -labeled protein doublet that might represent α and β tubulin was observed when binding to the stathmin fusion protein was performed at pH 6.8, but not when binding was performed at pH 8.0. Similarly, the anti-tubulin antiserum recognized a doublet only when binding to stathmin was performed at pH 6.8 in the presence of MgCl_2 , consistent with earlier studies (Curmi et al. 1997). Better binding at a lower pH (i.e., 6.8) has not been shown for other stathmin-binding proteins. In fact, experiments in this study suggest that stathmin may preferentially bind to a different set of

proteins at higher pH (i.e., 8.0 vs. 6.8). The dependence on pH could reflect many factors, including inherent differences among proteins, requirements for cofactors, or protein modifications such as phosphorylation. The weakness of the ^{35}S -labeled bands and the tubulin immunostaining may reflect the low affinity of the stathmin-tubulin interaction (Curmi et al. 1997) combined with the extensive washing employed in these studies.

Several other putative stathmin-binding proteins were isolated using the GST-stathmin fusion protein to probe Nb2- and PC12-cell extracts. Additional studies will be required to establish the identities of these proteins and their relationships to previously identified stathmin-binding proteins. The two prominent PC12 proteins migrating just below 30 kDa probably represent two or more isoforms of GST because the proteins were isolated using glutathione-Sepharose and recognized by an anti-GST antibody. The PC12 GST, which

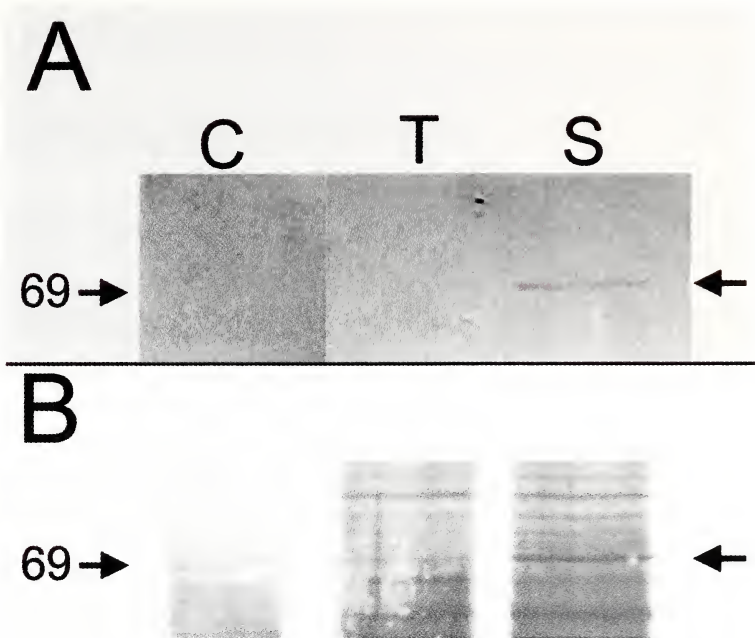


Figure 4.—³⁵S-labeled proteins in a cytosol fraction from Nb2 cells incubated at pH 8.0 with no additions (C), GST (T) or GST-stathmin (S). Proteins were isolated on glutathione-Sepharose and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and probed with anti-Hsp70 (A) or analyzed by autoradiography (B). In Fig. 4A, the arrow at right shows the position of a 70-kDa band recognized by the anti-Hsp70 antibody. In Fig. 4B, the arrow at right shows the position of a band that is more prominent in the GST-stathmin lane. The number at left shows the positions a 69-kDa standard.

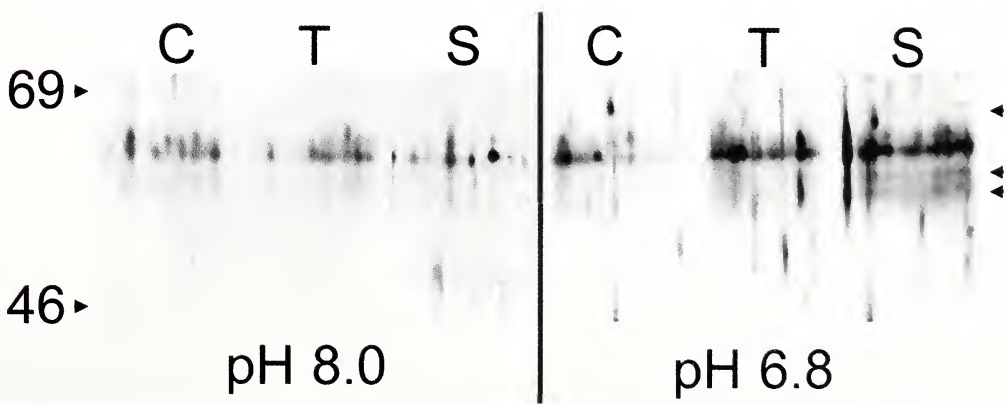


Figure 5.—Proteins in cytosol and membrane fractions from Nb2 cells incubated at pH 8.0 or pH 6.8 and 5 mM MgCl₂ with no additions (C), GST (T) or GST-stathmin (S). Proteins were isolated on glutathione-Sepharose, separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-tubulin. Arrowheads at right show the positions of proteins recognized by the anti-tubulin antibody, including a doublet precipitated by the GST-stathmin probe at pH 6.8. The numbers at left show the positions of molecular-weight standards.

may have been induced by mitogens in the serum (Nur-E-Kamal et al. 2000), approximated the amount of GST/GST-stathmin used to probe the extracts.

Overall, the results show that stathmin binds Hsp70 as well as BiP/Grp78 (Maucuer et al. 1995) and Hsc70 (Manceau et al. 1999). Thus, some of the growth-promoting and developmental actions linked to stathmin may involve interaction with Hsp70. Furthermore, the GST-stathmin fusion protein, which proved to be a good probe for stathmin-binding proteins, identified additional potential binding proteins. It seems likely that stathmin associates with multiple binding partners; consistent with the proposal that stathmin acts as an integrative, relay protein (Sobel 1991).

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