We have recently isolated the cDNA for the murine homologue of the stress-inducible phosphoprotein STI1 (also known as IEF SSP 3521 or p60). STI1 was previously shown to be 2-fold up-regulated in MRC-5 fibroblasts upon viral transformation and to exist in a macromolecular complex with heat shock proteins of the HSP 70 and 90 families. By peptide-sequencing we have identified the two heat shock proteins that bind to murine STI1 (mSTI1) as HSC 70 and HSP 84/86. We describe two separate binding regions within mSTI1 for the two heat shock proteins. In the presence of cell extracts, the N-terminal region of mSTI1 binds preferentially to HSC 70, whereas the C-terminal portion of the molecule promotes the binding of HSP 84/86. Heat treatment caused a strong induction of mSTI1 message without affecting the steady-state level of the protein significantly. In addition, heat treatment led to changes in the isoform-composition of mSTI1. pp90rsk, pp90rsk', and mitogen-activated protein kinase-activated protein kinase 2 were tested as possible STI1 kinases in vitro using recombinant mSTI1 as a substrate: only pp90rsk' was able to phosphorylate recombinant mSTI1. In vitro kinase assays using casein kinase II suggest serine 189 to be a likely phosphorylation site in mSTI1.

STI1 is a stress-inducible phosphoprotein first described in Saccharomyces cerevisiae, where it was implicated in mediating the heat shock response of some HSP 70 genes (3). Yeast STI1 (ySTI1) shares 42% amino acid identity with its human (hSTI1) and mouse cognates (mSTI1), the former one being previously named IEF SSP 3521 (4) or p60 (5). Furthermore, ySTI1 shows 38% amino acid identity to the STI1 homologue from soybean (gmSTI1) whose cDNA was recently cloned (6). Most recently the very C-terminal regions of ySTI1 and hSTI1 have been shown to be structurally related to Hip, a tetrastratic peptide repeats (TPR) motif containing protein that participates in the regulation of HSC 70 (7).

hSTI1 was shown to be up-regulated approximately 2-fold following viral transformation (4). Moreover, it was reported to be localized primarily to the nucleus in SV40-transformed MRC-5 fibroblasts, whereas in normal MRC-5 fibroblasts, localization to the Golgi apparatus and small vesicles was observed (4). The exact function of STI1 is still unknown, although the protein has been proposed to play an important role in cell proliferation or gene regulation (4).

The most notable sequence motif found in all STI1 homologues described so far is the so-called TPR which is believed to be important for the formation of macromolecular complexes (8). STI1 proteins from several species have been described to form complexes with stress proteins of the HSP 70 (5) and HSP 90 (5, 9) families. The abundance of the STI1-HSP 70-HSP 90 complex has led to the suggestion that these three proteins function interactively in an as yet undefined cellular context (5). Using a luciferase renaturation assay it was shown that in contrast to HSP 70 and HSP 90, hSTI1 does not possess any significant chaperoning activity (10). Moreover, in an in vitro reconstitution system using rabbit reticulocyte lysate to study the assembly of the chicken progesterone receptor, STI1 was identified as a transient component in this process without a defined molecular function (reviewed in Smith and Toft (11) and Johnson and Toft (12)).

At least three different stress-relevant MAPK-related pathways have been identified in mammalian cells so far (2). These are: 1) the classical p42/p44 pathway, named after extracellular signal-regulated kinase 1/2, which itself can be activated by stress (13) and lies upstream of the stress-inducible S6 kinase

1 The abbreviations used are: STI1, stress-inducible protein 1; gm STI1, mSTI1, ySTI1, and hSTI1, soybean, murine, yeast, and human STI1, respectively; IEF SSP 3521, isoelectric focusing sample spot 3521; HSP, heat shock protein; GST, glutathione S-transferase; NLS, nuclear localization signal; MAPK, mitogen-activated protein kinase; MAPKAP kinase, MAPK-activated protein kinase; TPR, tetrasstratic peptide repeats; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; HSC, heat-shock cognate.

2 G. L. Blatch, V. Kundra, M. Lässle, T. Takatori, and B. R. Zetter, manuscript in preparation.
pp90rsk, also known as MAPKAP kinase 1 (13, 14); 2) the p38/HOG1 pathway, which leads to activation of MAPKAP kinase 2 and which is responsible, e.g., for the changes in the phosphorylation pattern of HSP 25 (15); and 3) the Jun N-terminal kinase/stress-activated protein kinase pathway which leads to the activation of members of the Jun transcription factor family (16). Extracellular signal-regulated kinase 1/2, pp90rsk, and Jun N-terminal kinase 1 are known to translocate into the nucleus where they affect the phosphorylation state of their nuclear targets (2). In the case of the p38/HOG1 pathway, it is p38 itself rather than the further downstream effector MAPKAP kinase 2 that translocates into the nucleus. An additional stress-relevant protein kinase which has not been implicated in classical MAPK pathway patterns is S6 kinase pp70s6k (17). This kinase exists in two isoforms (70 and 85 kDa) within the cell and has been shown to be activated by heat stress (13, 14).

In this report, we examine the interactions of mSTI1 with HSP 90 and HSC 70. We have identified the HSP binding partners of mSTI1 by peptide sequencing as HSC 70 and HSP 84/86. We have further characterized their binding interaction in more detail utilizing a set of glutathione S-transferase (GST) fusion proteins comprising different parts of the mSTI1 molecule. Furthermore, we describe the effects of heat treatment on mSTI1 mRNA and protein levels in NIH 3T3 cells. Besides the existence of a nuclear fraction of mSTI1, we have found most of the protein to be cytosolic. Finally using different fusion proteins of mSTI1 as substrates in in vitro phosphorylation reactions with different kinases, we offer an explanation for the observed localization of the nuclear fraction of this protein, as well as linkage to a specific stress-relevant signal transduction pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**

Protein A- and protein G-agarose were obtained from Boehringer Mannheim. Glutathione-agarose was from Sigma. Tran<sup>32</sup>P-ATP (6000Ci/mmol) was purchased from ICN (Costa Mesa, CA). [γ-<sup>32</sup>P]ATP (6000Ci/mmol) was purchased from Amer sham Corp. Casein kinase II and casein kinase II reaction buffer were from New England Biolabs (Beverly, MA). Partially purified fractions of MAPKAP kinase 2 and pp90s6k were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Recombinant pp70s6k in the form of GST fusion protein as well as the control substrate GST-S6 were kindly provided by J. Blenis (Harvard Medical School, Boston). Recombinant murine HSP 25 was from StressGen (Victoria, BC, Canada). Bovine brain HSC 70 and HSP 90 were purchased from Sigma. Rabbit skeletal muscle actin was a gift from P. Janney (Brigham’s and Women’s Hospital, Boston).

**Antibodies**

Preparation of STI1 Specific Antibody SF1—Immunization of rabbits and collection of bleed samples was performed by ARCH (Arch Resources Children’s Hospital, Boston) according to standard procedures (18). Briefly, female New Zealand White Rabbits (5–6 pounds) purchased from Charles River Laboratories (Wilmington, MA) were injected intradermally with 250 μg of mSTI1 purified as a GST fusion protein comprising the 334 C-terminal amino acids (GST-C334 in Fig. 4). First and second boost injections were given 4 and 8 weeks after the initial injection. Blood samples were collected from the marginal ear vein, for testing samples, 10 days after injection, for production samples normally around day 14. Purification of the IgG fraction followed the low salt purification method on protein A-agarose (Boehringer Mannheim) according to Harlow and Lane (18). Additionally antibodies specific for the GST portion of the antigen were routinely removed by adsorption to a matrix prepared from Escherichia coli expressing GST (18). Affinity purification of SF1 was performed on a CNBr-activated Sepharose 4B matrix (Pharmacia Biotech Inc.) to which highly purified GST-C334 was coupled.

Other Antibodies—An HSP 70 specific antibody that recognizes both HSP 72 and 73, clone W27 (Oncogene, Uniondale, NY) was used in Western blot analysis. HSC 70-specific rat monoclonal antibody 1B5 (StressGen, Victoria, BC, Canada) was used for immunoprecipitation experiments. HSP 90 antibody AC88 was kindly provided by D. Toft (Mayo Clinic, Rochester, MN). HSP 25 specific antibody SPA-801 was purchased from StressGen. Anti-actin mouse monoclonal C4 was from Boehringer Mannheim. Horseradish peroxidase-coupled secondary rabbit and mouse specific antibodies were from Amersham Corp.

**Preparation of GST-mSTI1 Fusion Proteins**

Plasmids—Plasmids pSK1400 and pCR1300 will be described elsewhere. They contain as inserts the 3' and 5' ends, respectively, of the cDNA encoding mSTI1. Recombinant proteins were produced as GST fusion proteins using the pGEX3 X E. coli expression vector (Pharmacia). Plasmid pGEXX1400 (encoding GST-C334) was constructed by ligating in the correct orientation the 1400-base pair EcoRI insert fragment of pSK1400 into EcoRI digested pGEXX3. Plasmid pGEXXX2000 (encoding the 670-base pair EcoRV-BglII fragment of pCR1300 to the 6300-base pair Smal-BglII fragment of pGEXX3X1400). Plasmids pGEXX850 (encoding GST-N880) and pGEXX3700 (encoding GST-N217), pGEXX3550 (encoding GST-N174), and pGEXX2200 (encoding GST-N56) are derivatives of pGEXX3X2000 in which the HindIII-NcoI, BglII-NcoI, SmaI-NcoI, and Stul-NcoI fragments, respectively, have been deleted. Recircularization of these deletion derivatives was achieved by creating blunt ends using T4 DNA polymerase (Boehringer Mannheim) followed by self-ligation.

**Preparation of Cell Lysates, Protein Concentration Determination, SDS-PAGE, and Western Blotting**

Whole cell lysates were prepared on ice by washing cells with PBS before scraping them directly into buffer A (30 mM HEPES, pH 7.3, 5 mM MgCl₂, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml pepstatin, 0.8% Nonidet P-40, 200 mM NaCl). Any remaining cell debris was removed by a 20-min centrifugation at 4°C. The supernatant was stored at −80°C. SDS-PAGE was performed according to Laemmli (21), Western blotting to nitrocellulose (Schleicher & Schuell, Keene, NH) was performed according to Towbin et al. (22). As a detection system for Western blot analysis, the enhanced chemiluminescence system from Amersham was used with horseradish peroxidase-coupled secondary antibodies. Where noted, blots were used twice with removal of previously bound antibody by incubation at 55 °C for 1 h in buffer S (100 μM β-mercaptoethanol 2% SDS, 62.5 mM Tris-Cl pH 6.7).

**Glutathione-Agarose Binding Assays**

GST fusion proteins were coupled to glutathione-agarose at 0.25 μl. Removal of unbound fusion protein by washing three times with PBS was followed by incubation in the presence of 70- and 90-kDa agarose-beads with 150 μg of extract proteins for 1 h at 4 °C. The beads were then washed another three times with PBS to remove nonspecifically bound extract proteins. After solubilization in SDS sample buffer, proteins were usually separated by 12.5% SDS-PAGE.

**Peptide Sequencing**

5 × glutathione-agarose binding assays with either the GST-mSTI1 fusion protein GST-N217 (for the 70-kDa protein) or GST-C334 (for the 90-kDa protein) were performed as described above. After separating the assay proteins corresponding to 1 and 4 times the amounts of assay beads in parallel on a 10% SDS-PAGE, the gel was split, and the 1 time lane was stained as a control with Coomassie while the 4 times lane was electrophoblated onto nitrocellulose. Proteins on the nitrocellulose were detected with Ponceau S. 70- and 90-kDa bands were excised from the membrane. The membrane-eluted proteins were then submitted to in situ digestion with Lys-C (70 kDa) or trypsin (90 kDa), and the resulting peptide mixture was separated by HPLC using a Vydac C18 (2.1 × 150 mm) reversed-phase column on a Hewlett-Packard 1090 HPLC with a 1040 diode array detector. The sequence of selected peaks was determined by automated Edman degradation on an ABI model 477A protein sequencer (Applied Biosystems, Foster City, CA). Strategies for peak selection, reversed-phase separation, and protein microsequencing have been described previously (23).

**Heat Treatment of NIH 3T3 Cells**

NIH3T3 cells were grown in Dulbecc’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C and 10% CO₂ until they reached 90% confluence.
Analysis of Proteins—Control cells (0 min, 42 °C) were collected immediately after trypsinization followed by two washes with PBS. Standard whole cell lysates were prepared using buffer A. For heat treatment, cells were transferred to an incubator at 42 °C and incubated for 0, 30, 60, and 150 min before extract preparation. Same amounts of total protein (30 μg) were separated by 12.5% SDS-PAGE (21). The proteins were then transferred to nitrocellulose and the immunoblot was probed with the anti-mSTI1-specific antibody SF1 before detection of the secondary horseradish peroxidase-labeled antibody with Amersham’s ECL system. After stripping, the nitrocellulose was reprobed with HSP 70-specific antibody W27 as a positive control.

Analysis of RNA—After heat shock, total cellular RNA was prepared according to Chomczynski and Sacchi (24). The mRNA was prepared by passing 300 μg of total RNA through an oligo(dT) column (Stratagene, La Jolla, CA). Half of the mRNA for each time point was used for Northern analysis. Briefly, mRNA was resolved by 1% agarose-gel electrophoresis under denaturing conditions (2.2 m formaldehyde) and blotted onto a Hybond N+ membrane (Amersham) by the capillary method (25). Northern hybridization was carried out using a 32P-labeled (Random Primed DNA labeling kit, Boehringer Mannheim) 750-base pair BglII-BstXI cDNA fragment as a probe. Before reprobing the blot with a β-actin control-probe it was stripped of the mSTI1 probe by boiling for 10 min in 0.5% SDS. The mouse β-actin probe was polymerase chain reaction amplified using the mouse β-actin amplimer primer set from Clontech (Palo Alto, CA). RNA size markers were from Promega (Madison, WI).

Two-dimensional Protein Gel Electrophoresis

Cell extract proteins were resolved by two-dimensional electrophoresis using a modification of the procedure originally described by O’Farrell (26). A 3:1 ratio of broad (pH 3.5–10) and narrow (pH 6–8) range ampholytes was used for the separation in the first dimension. Each ampholyte was prepared as a 1:1 mixture from two different sources (Bio-Rad and Pharmacia). Carboxymethylated protein standards (Pharmacia) were used in the first dimension to allow exact alignment of control and heat-treated STI1 isoforms.

In Vitro Phosphorylation Experiments

Casein Kinase II Assay—Casein kinase II assays were performed in a 20-μl reaction volume with the different mSTI1-GST fusion proteins at a substrate concentration of 5 μM. The casein kinase reaction buffer was supplemented with 200 μM ATP and [γ-32P]ATP to a final specific activity of 400 μCi/μmol. 100 units of casein kinase II were routinely used per reaction (7 μg/ml). Incubation was for 1 h at 30 °C. The reaction tubes were then transferred on ice and 1 volume of a 50% glutathione agarose-beads suspension in PBS was added to each tube. After incubation for 10 min on ice, 1 μl of PBS was added, and the beads were collected by centrifugation. This wash step was repeated three times until there was no radioactivity detectable in the supernatant above background level. The beads were finally resuspended in 1 × Laemmli sample buffer and separated by SDS-PAGE on a 12.5% gel. In reactions where a possible stimulatory effect of HSP 90 on casein kinase II activity was tested, the mSTI1-GST fusion protein concentration was lowered to 2.5 μM. HSP 90 or, as a negative control, HSC 70 were used at a 100/1 ratio with 4 μg/ml casein kinase II. Quantitation of the substrate phosphorylation was performed with a PhosphoImaging system (Molecular Dynamics) using the Image Quant program.

MAPKAP Kinase 2 Assay—Substrates were used in a final reaction volume of 20 μl at 2.5 μM. MAPKAP kinase 2 reaction buffer contained 50 mM β-glycerol phosphate, 0.1 mM EDTA, 10 mM magnesium acetate, pH 7. Reactions were supplemented with ATP to 200 μM. The specific activity of [γ-32P]ATP was 400 μCi/μmol ATP. Routinely, 0.2 unit of enzyme was used in a 30-min reaction at 30 °C. Substrate isolation for GST fusion proteins followed the procedure described for casein kinase II. The reactions containing HSP 25 and HSC 70 were treated differently so far as the substrates were immunoprecipitated by adding 10 μl of antibodies SPA-801 and 1B5, respectively, followed by 20 μl of a 50% suspension of protein A- and G-agarose, respectively.

pp70S6k and pp90Rsk Assays—0.3 unit of pp90rsk or pp70s6k at 0.25 μM was used under the same assay conditions as described for the MAPKAP kinase 2 assay with the substrates at 5 μM. Isolation of the substrates followed the procedure outlined for casein kinase II.

Immunofluorescence Microscopy

All procedures were carried out at room temperature. Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with PBS, containing 0.5% bovine serum albumin, 0.25% Triton for another 15 min and then incubated with either affinity-purified SF1 antibody or unspecific rabbit IgG (Sigma) diluted in PBS, 0.5% bovine serum albumin, ovalbumin.

RESULTS

Interaction of mSTI1 with HSPs of 70 and 90 kDa—Murine STI1 was cloned from an M27 Lewis lung cDNA expression library encoding a protein with a molecular mass of 63 kDa (GenBank™ accession no. U27830). A GST-STI1 fusion comprising the nucleotides corresponding to the C-terminal 334 amino acids of mSTI1 was constructed, and the resulting fusion protein (GST-C334 in Fig. 2) was used to prepare polyclonal antibody SF1 specific for mSTI1. The antibody recognizes a single protein band in the 60-kDa range in whole cell mouse extracts (for an example, see Fig. 5, panel A, lane 1) and cross-reacts with the rat and human antigen (not shown). In order to identify proteins associated with mSTI1, we performed immunoprecipitation experiments using SF1 and metabolically labeled M27 cell extracts (Fig. 1). When used in low amounts SF1 precipitates the expected specific band in the 60-kDa range (lane 3) that is completely lacking in the preimmune control precipitation (lane 1). By using higher amounts of SF1 (lane 4), but not preimmune serum (lane 2), two additional specific bands coprecipitate with mSTI1, one in the 70-kDa range, the other one, which actually consists of a doublet, in the 90-kDa range. Because HSP 90 is known to run as a doublet in this size range, we performed Western blot analysis using an antibody specific for HSP 90. The identity of the 90-kDa protein with HSP 90 family members was confirmed, and subsequently the 70-kDa protein was identified in a similar way as a HSP 70 family member (data not shown).

Differential Binding of the HSP 70 and HSP 90 Molecules from M27 Cell Extracts to GST Fusion Proteins Comprising N- or C-Terminal Regions of mSTI1—The structural domains of STI1 that are responsible for the binding to HSPs 70 and 90 are currently not known. Therefore, we studied the binding behavior of the 70- and 90-kDa HSP proteins toward a set of GST-STI1 fusion proteins comprising different parts of mSTI1 (Fig. 2). Using these different fusion proteins we performed glutathione-agarose binding assays with metabolically labeled M27
Beads were then incubated with 150 μl of lane 5 fusion protein GST-C334 (binds specifically to GST-C334 and GST-543 (not HSP70). The protein migrating directly below HSP70 that is shown to bind differentially to murine STI1, we obtained the partial TPR motifs which is highly conserved among HSP 70 members. Seven peptides were derived from the 70-kDa protein that represented discrete regions of the mSTI1 protein. As can be seen in the assays using the N-terminal fusion proteins GST-N217 (lane 2) or GST-N302 (lane 3), only the 70-kDa HSP and not the 90-kDa HSP bound to the N-terminal region. In contrast, the C-terminal fusion protein GST-C334 (lane 4) bound only HSP 90 and not HSP 70. The protein migrating directly below HSP 70 that binds specifically to GST-C334 and GST-543 (lanes 4 and 5) most likely represents extract endogenous mSTI1.

Identification of the HSPs That Bind to Murine mSTI1 as HSP 70 and HSP 90—To identify more precisely the HSPs that bind differentially to murine STI1, we obtained the partial amino acid sequence of the murine HSP 70 and HSP 90 family members. Specifically, we isolated the HSP molecules from M27 Lewis lung carcinoma cell extracts, using the glutathione-agarose bead-coupled fusion protein GST-C334. The sequence numbering of amino acids refers to murine HSP 70 (SwissProt accession no. M18186) to which all peptides except 1 and 5 showed 100% identity. Peptide 1 includes two unidentified amino acids that are denoted as Xs, and peptide 5 contains two amino acids that do not match HSP 84 in positions 457 and 459.

| Peptide | Amino acid | Sequence |
|---------|------------|----------|
| 1       | 5–16       | PAVGIDLGTYS |
| 2       | 37–49      | TIPSYVAFTDIER |
| 3       | 57–70      | NQVAMNPTNTVPEDA |
| 4       | 89–100     | HWPFWVVDNAGR |
| 5       | 113–126    | SYFPEEVSSMLTK |
| 6       | 160–171    | DACTIALGNVR |
| 7       | 172–197    | LNPETAAAAYGLDK |
| 8       | 221–235    | STAGDTHLGGEDFDN |
| 9       | 300–311    | ARFEELNADLFR |
| 10      | 329–342    | SQINHDVLVGGSTR |
| 11      | 349–357    | LLDFFSNKG |
| 12      | 362–383    | SINPDEAVAYGAQQAILSDD |
| 13      | 610–629    | LYGQAXGMPGMXGKFPG |

Table I

| Peptide | Amino acid | Sequence |
|---------|------------|----------|
| 1       | 2–19       | FEEVHGEESVEVTFAXA |
| 2       | 180–195    | VILMLKEDQTELEYER |
| 3       | 291–305    | NPDDITQEYEGFYK |
| 4       | 428–434    | FYEAFSK |
| 5       | 456–468    | YQTIQGDSPMTSL |
| 6       | 481–492    | SLYVITQESK |
| 7       | 652–674    | DUUYLLFSTALLSSGFSLEDPQ |

Table II

| Peptide | Amino acid | Sequence |
|---------|------------|----------|
| 1       | 2–19       | FEEVHGEESVEVTFAXA |
| 2       | 180–195    | VILMLKEDQTELEYER |
| 3       | 291–305    | NPDDITQEYEGFYK |
| 4       | 428–434    | FYEAFSK |
| 5       | 456–468    | YQTIQGDSPMTSL |
| 6       | 481–492    | SLYVITQESK |
| 7       | 652–674    | DUUYLLFSTALLSSGFSLEDPQ |
The differential binding pattern of HSC 70 to the different GST-mSTI1 fusion proteins can be reconstituted in the absence of extract proteins. Different GST-mSTI1 fusion proteins were coupled at 0.25 μM to glutathione-agarose. They were then incubated with HSC 70 or HSP 90 at 0.025 μM (top and middle panels) and actin (bottom panel) at 0.25 μM for 1 h at 4°C. Further sample treatment was the same as described for Fig. 3. Binding assays were performed with GST (lane 1), GST-N56 (lane 2), GST-N174 (lane 3), GST-N217 (lane 4), GST-C354 (lane 5), and GST-543 (lane 6). The actual amount of substrate added into the assay is shown in lane 7 for HSC 70 and HSP 90 (2.6 and 3.4 μg, respectively). In the case of actin, one-fifth of the substrate added into the assay is shown (3.1 μg). HSC 70, HSP 90, and actin were visualized after Western blotting and immunodetection (with antibodies W27, AC88, and C4, respectively) as described under “Experimental Procedures.”

Heat Inducibility of Murine STI1—The association of STI1 with HSPs to murine STI1, we attempted to reconstitute the differential binding behavior of the different GST fusion proteins toward purified HSP 90 and HSC 70. Both purified HSPs bound to GST-543 (Fig. 4, top and middle panels, lane 6). Yet, the ratio of bound to input substrate was clearly higher for HSC 70 (top panel, lanes 6 and 7) than for HSP 90 (middle panel, lanes 6 and 7). Moreover, while purified bovine HSC 70 showed the expected stronger binding to the N-terminal portion of the mSTI1 molecule (Fig. 4, top panel, compare lanes 3 and 4 with 5), purified bovine HSP 90 failed to show the expected preference for the C-terminal portion of the molecule in the absence of cell extract-derived proteins (Fig. 4, middle panel). Finally under these conditions HSP 90 did bind to the GST control (Fig. 4, middle panel, lane 1). In contrast the GST control did not bind HSC 70 under the same conditions. The experiment in Fig. 4 additionally revealed no binding of HSC 70 to the fusion protein comprising only the first 56 amino acids of murine mSTI1 (GST-N56, Fig. 4, top panel, lane 2). None of the fusion proteins bound the control substrate actin (Fig. 4, bottom panel) above background levels.

Heat Inducibility of Murine STI1—The association of STI1 with HSPs (5, 9) (this study) is the second important link of this molecule to stress-relevant cellular events. Human STI1 was originally detected due to a 2.2-fold up-regulation of the protein upon viral transformation of fibroblasts, with mRNA levels being increased even more drastically (4). For the yeast (3) and soybean (6) homologues, heat inducibility of STI1 mRNA has also been shown. The influence of heat treatment on mSTI1 protein (Fig. 5) and mRNA levels (Fig. 6) was therefore investigated. As can be seen in NIH 3T3 whole cell extracts, there is no effect of 30-, 60-, or 150-min incubation at 42 °C on mSTI1 steady-state protein levels (Fig. 5, panel A, lanes 2, 3, and 4) when compared to the control level at 37 °C (Fig. 5, panel A, lane 1). As a positive heat induction control, the Western blot of Fig. 5, panel A, was reprobed with an antibody that was able to detect the strongly heat-inducible protein HSP 70 (Fig. 5, panel B). The influence of heat treatment on mSTI1 mRNA levels can be seen in Fig. 6. NIH 3T3 cells kept for 60 min at 42 °C (Fig. 6, panel A, lane 3) show a drastic increase in STI1 mRNA as compared to 37 °C control levels (Fig. 6, panel A, lane 1). This effect is even more drastic after 150 min at 42 °C (Fig. 6, panel A, lane 4). The effect of 30-min heat treatment on STI1 mRNA levels (Fig. 6, panel A, lane 2) must be seen in the context of somewhat higher amounts of total mRNA in this particular lane, as indicated by the reprobing of the same blot with a β-actin-specific probe (Fig. 6, panel B).

Heat Treatment Changes the Isoform Composition of mSTI1—In addition to investigating the influence of heat stress on the expression of mSTI1, we also studied potential modifications of the protein. Fig. 7 shows the detection of STI1 in NIH 3T3 cell extracts by Western blot analysis with antibody SF1 after separation by two-dimensional gel electrophoresis. In the absence of heat treatment, mSTI1 migrated as a single major isoform (Fig. 7, top panel, spot 0). The bottom panel of Fig. 7 shows a dramatic change in isoform composition after 10 min of heat treatment. While the amount of isoform 0 decreases, one more basic (spot –1 in Fig. 7, bottom panel) and two more acidic (spots +1 and +2 in Fig. 7, bottom panel) isoforms were induced. A small amount of the more acidic isoform +1 was already detectable under non-heat-stress conditions (Fig. 7, top panel). These results suggest that a subpopulation of mSTI1 does undergo modification upon heat treatment.

Phosphorylation of mSTI1 by Casein Kinase II in Vitro—One possible modification that could account for the stress-induced change in STI1 isoform composition after either viral transformation of human cells (4) or heat treatment of murine cells.
binding domains and phosphorylation of mSTI1

### FIG. 7. mSTI1 isoform composition changes upon 10 min of heat treatment.
Western blot analysis of the STI1 isoform composition after two-dimensional gel analysis of 200 μg of NIH 3T3 whole cell extracts separated according to the method of O’Farrell (26) with minor modifications as described under “Experimental Procedures.” The extract separated in the top panel of the figure was untreated, the one used for the bottom panel was prepared from cells that were incubated for 10 min at 42 °C. After aligning the two blots, the most prominent spots were labeled, with the most abundant spot under nontreatment conditions getting number 0, spots located toward the more acidic region of the gel getting increasing positive numbers and spots located in the more basic region of the gel getting negative numbers.

### FIG. 8. Potential phosphorylation sites within mSTI1.
The amino acid motif potentially recognized by casein kinase II is STX-X-D/E (underlined) with the additional presence of acidic amino acids C-terminal to the phosphate acceptor (underlined and marked with an asterisk (*) in Fig. 8). Other kinase recognition motifs are just indicated by boxes and described in more detail in the text. The beginning (dashed line) and end (solid line and arrow) of the STI1 sequence in the different GST fusion proteins is also indicated, as is the putative NLS from amino acids 222–239 (gray shaded area with labeled lysines).

This study (Fig. 7) would be differential phosphorylation of the STI1 molecule.

We therefore performed in vitro kinase assays using our mSTI1-GST fusion proteins as substrates. Two groups of kinases were of particular interest to us, stress-related kinases and kinases that are known to phosphorylate heat shock proteins. Casein kinase II has been shown previously to be involved in the phosphorylation of HSP 90. HSP 90 is phosphorylated by casein kinase II in vitro and in vivo (28), and both proteins form a complex in cell lysates under low salt conditions (29). There are six potential casein kinase II sites within the amino acid sequence of mSTI1 (Fig. 8). It is important to note that the GST sequence itself already contains two potential phosphorylation sites for casein kinase II (not shown). Three of the different substrates used in Fig. 9 (GST, lane 1; GST-N302, lane 4; and GST-543, lane 6) do show specific incorporation of 32P during the kinase reaction. Densitometric quantitation of the bands in Fig. 9 showed that there is approximately 2–3-fold stronger phosphorylation of GST-N302 or GST-543, respectively, when compared to GST (data not shown). The most interesting potential phosphorylation site of the three shared sites in GST-N302 and GST-543 is the most C-terminal one (marked with an asterisk (*) in Fig. 8). It contains a short stretch of acidic amino acids C-terminal to the phosphate-accepting serine 189. Such a sequence motif is known to dramatically increase the probability for phosphorylation by casein kinase II (30). A closer look at the amino acid sequence surrounding this particular potential phosphorylation site led us to the identification of a putative bipartite nuclear localization signal (NLS) (31) between the two stretches of basic amino acids (underlined). The bottom part compares amino acids 221–239 of mSTI1 including the putative NLS and the homologous regions of STI1 molecules from other species. e, viral; s, Xenopus; h, human; y, yeast; m, mouse; gm, soybean.

### FIG. 9. In vitro kinase assay with different GST-mSTI1 fusion proteins and casein kinase II.
Autoradiogram of the reaction products of casein kinase II in vitro kinase assays with different GST-mSTI1 fusion proteins used as substrates. Substrates included in the standard assay (see “Experimental Procedures”) were GST (lane 1), GST-N56 (lane 2), GST-N174 (lane 3), GST-N217 (lane 4), GST-C334 (lane 5), and GST-543 (lane 6). The migration position of the different fusion proteins is marked (>), and both bands detectable in the autoradiogram are due to phosphorylation of minor contaminants in the fusion protein preparations.

| molecule | NLS | spacer |
|----------|-----|--------|
| GST-C334 | X-X |        |
| GST-N174 | X-X |        |
| GST-N217 | X-X |        |
| GST-N302 | X-X |        |
| GST-543  | X-X |        |

**Localization of mSTI1 in NIH 3T3 Cells**—We examined the cellular distribution of murine STI1 by indirect immunofluorescence in NIH 3T3 cells using an antibody raised against a C-terminal peptide (amino acids 370–381). We observed apparent nucleolar staining as well as diffuse cytosolic staining (not shown). Using affinity purified antibody ySTI1, however, we observed only cytosolic staining for mSTI1 in NIH 3T3 cells (Fig. 11). We were never able to detect localization of cytosolic mSTI1 to any particular organelles, as has been described for the human homologue (4).
phosphorylation motifs of kinases known to be activated by heat stress (Fig. 8, boxed sequences) such as the S6 kinases or MAPKAP kinase 2 (14, 33). Apart from two R[X]-S sites (amino acids 142–145 and amino acids 476–481), which are part of the phosphorylation motif in the pp70Ras kinase preparations showed strong phosphorylation activity toward the positive control substrate GST-S6 (Fig. 13, lane 4). The unidentified band migrating between 50 and 64 kDa detectable in the HSP 25 kinase assay (lane 4) most likely represents a minor contaminant in the HSP 25 preparation that binds strongly to the protein A-agarose or cross-reacts with the antibody and is a substrate for MAPKAP kinase 2. HSC 70 was another substrate that was phosphorylated by MAPKAP kinase 2 under our assay conditions, although clearly less than was HSP 25 (Fig. 12, lane 5).

We also tested the possibility of in vitro phosphorylation of mSTI1-GST fusion proteins by the two S6 kinases. Of these, pp90Ras was clearly able to phosphorylate GST-N56 (Fig. 13, panel B, lane 2), whereas pp70Ras did not phosphorylate any of the mSTI1-GST fusion proteins (Fig. 13, panel A). Again, the kinase preparations showed strong phosphorylation activity toward the positive control substrate GST-S6 (Fig. 13, panels A and B, lane 7).

**DISCUSSION**

Earlier studies on the interactions of heat shock proteins showed the existence of a complex consisting of HSP 70, HSP 90, and STI1 that is conserved from yeast to vertebrates (9, 5). This complex can include, dependent on the isolation procedure, several other proteins (reviewed in Smith and Toft (11)). All of these studies identified STI1 as a component of HSP 70 or HSP 90 immune complexes, and the identification of the HSPs in individual experiments was based mostly on their antigenicity. Exact identities and interaction patterns of molecules that participate in the formation of such macromolecular complexes are difficult to establish under these circumstances.

In the current study we investigated interactions within the above mentioned complex by focusing on the murine homologue of the tetratrico peptide repeat protein STI1 and derived GST fusion proteins thereof. We unambiguously identify the HSPs that interact with mSTI1 as HSC 70 and HSP 84/86 by peptide sequencing. Within cell lysates the interaction of mSTI1 with HSC 70 is restricted to the N-terminal part of the molecule, whereas the C-terminal part of the molecule is involved in binding of HSP 90. The distribution of TPR motifs in an N-terminal and a more C-terminal cluster within murine STI1 is intriguing in this context and implies a possible bridging function for STI1 in the complex formation with the two heat shock proteins. Our binding studies indicate that the two N-terminal motifs are involved in HSC 70 binding, with the most N-terminal motif alone being unable to bind HSC 70. In vitro reconstitution experiments show that preferential binding of HSC 70 to the N-terminal region of mSTI1 does not require the addition of exogenous cellular proteins, whereas HSP 90 binding restricted to the C-terminal region of the mSTI1 molecule occurs only in the presence of cell extract proteins. The nature of the extract proteins that restrict HSP 90 binding to the C terminus of mSTI1 is currently unknown. The rationale for using GST fusion proteins in our study has been outlined above; it cannot, however, be excluded that some of the observed interactions in

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**FIG. 11.** Localization of mSTI1 in NIH 3T3 cells as determined by indirect immunofluorescence with affinity purified antibody SF1. Paraformaldehyde-fixed cells were permeabilized with Triton (0.25%), and after incubation with STI1 specific affinity purified antibody SF1, antigens were localized with the help of fluorescein isothiocyanate-conjugated rabbit-specific secondary antibodies.

**FIG. 12.** MAPKAP kinase 2 in vitro kinase assays with different GST-mSTI1 fusion proteins, HSP 25, and HSC 70. Autoradiogram of the reaction products of MAPKAP kinase 2 in vitro kinase assays with different substrates. Substrates included in the standard assay (see “Experimental Procedures”) were GST (lane 1), GST-N502 (lane 2), GST-543 (lane 3), murine HSP 25 (lane 4), and bovine HSC 70 (lane 5). The migration position of HSP 25 and HSC 70 is indicated. Molecular mass markers indicated on the left, from top to bottom: bovine serum albumin, glutamic dehydrogenase, alcohol dehydrogenase, carbonic anhydrase, myoglobin, and lysozyme.

**FIG. 13.** In vitro kinase assay with different GST-mSTI1 fusion proteins and pp70Ras (panel A) or pp90Ras (panel B). Autoradiogram of the reaction products of in vitro kinase assays with pp70Ras (panel A) or pp90Ras (panel B) using different GST-mSTI1 fusion proteins as substrates. Substrates included in the standard assay (see “Experimental Procedures”) were GST (lane 1), GST-N502 (lane 2), GST-N174 (lane 3), GST-302 (lane 4), GST-C334 (lane 5), GST-543 (lane 6), and GST-S6 (lane 7).
our fusion protein binding assays are due to improperly folded GST fusion proteins, as it is well known that molecular chaperones do bind to non-native state proteins (1).

Although heat stress causes a significant increase in mSTI1 mRNA levels, we find that steady-state protein levels are not changed after heat stress. A similar discrepancy in the heat inducibility of mRNA when compared to protein levels, has been described for hSTI1 upon viral transformation (4). We cannot exclude a weak induction of the protein upon stress as it was described by others (4), yet we do not see stress inducibility as its most prominent feature since the protein is quite abundant under nonstress conditions.

The identification of stress-related kinases possibly involved in the phosphorylation of STI1 is still at an early stage. However, the observation of shifts in the isoform composition of STI1 to more acidic forms after viral transformation (4) as well as after heat shock suggests the stress-induced phosphorylation of a STI1 subpopulation. We have set our initial focus on in vitro kinase assays using GST fusion proteins that comprise different parts of the mSTI1 molecule. Although mSTI1 does contain a potential phosphorylation site for MAPKAP kinase 2, neither the full-length GST fusion protein nor the smaller fusion proteins were phosphorylated in vitro by this kinase. We are aware of the fact that the site in question could be inaccessible due to folding artifacts of the fusion proteins and therefore cannot rule out completely that STI1 is a possible substrate for this kinase. Our results do, however, suggest that HSC 70 is a possible substrate for MAPKAP kinase 2 in vitro. In vitro kinase assays with the two S6 kinases indicate that neither of the two R-X-S-S sites within murine STI1 is easily phosphorylated by these enzymes. The only mSTI1 substrate phosphorylated by pp90rsk is GST-N56. There seems to exist some variability in substrate specificity of pp90rsk (32). Two catalytic domains within pp90rsk are known, a protein kinase A domain (target consensus R-R-X-S) and a phosphorylase b kinase domain (target consensus R-X-X-S) (32). The most inclusive description of a potential pp90rsk phosphorylation site suggests that pp90rsk shows a strong preference for serine C-terminal to arginine (34). Examination of the STI1 amino acid sequence reveals only one additional site, apart from the two R-X-S-S sites, that matches this description (S-N-R-S, amino acids 42–45). It is included in GST-N56 and is found at the very end of a stretch of 10 amino acids that are identical between all STI1 homologues described so far. Currently we do not know if it is indeed serine 45 that gets phosphorylated within GST-N56 by pp90rsk. Furthermore, other GST-STI1 fusion proteins that contain the amino acids of GST-N56, i.e. GST-N217, GST-N302, and GST-543 were not phosphorylated by pp90rsk in our experiments. As mentioned previously, these results may be influenced by the three-dimensional structure of the different fusion proteins.

The intracellular localization of STI1 appears to follow a rather complex pattern. It has been shown for the human homologue that, dependent on the cell type under investigation, the protein localizes either primarily to the nuclear/nucleolar compartment or to distinct organelles within the cytosol (vesicles or Golgi apparatus). In contrast, although we did observe nucleolar staining when using an antibody prepared against an internal peptide in the mSTI1 molecule, diffuse cytoplasmatic staining was the most prominent pattern seen when cells were stained using an affinity-purified polyclonal antibody.

The results of our in vitro phosphorylation experiments with casein kinase II together with the amino acid composition of the different potential casein kinase II sites within the mSTI1 sequence strongly suggest, although they do not prove, that serine 189 can be phosphorylated by this kinase.

Although STI1 has not been noted previously to contain a nuclear localization signal (4), we found a sequence motif within mSTI1 that corresponds to a nuclear localization signal of the bipartite type (31). Moreover, this potential NLS is found in all STI1 molecules described so far, with the exception of the yeast homologue.

The close proximity of an in vitro casein kinase II phosphorylation site to the potential NLS within mSTI1 suggests that the rate of nuclear entry of STI1 might be regulated by phosphorylation as it has been shown for other proteins (35). We were not able to identify casein kinase II in our immunoprecipitates obtained with antibody SF1 (data not shown), arguing against a complex of HSP 90, casein kinase II, and mSTI1. It has also been reported that HSP 90 enhances the kinase activity of casein kinase II (29) in studies that examined the in vitro phosphorylation of a peptide substrate. This view has recently been challenged (36). In our studies that examined casein kinase II phosphorylation of GST-STI1 fusion proteins in the presence of up to 100-fold excess amounts of HSP 90, we were unable to detect any stimulation of casein kinase II activity by HSP 90 (data not shown).

The association of STI1 in a heterocomplex with HSP 90 and HSP 70 is so far the most intriguing finding with regard to the function of STI1. This heterocomplex has been studied mostly in terms of HSP 90's role in the activation of steroid receptors (11, 12). However the function of this heterocomplex is most likely diverse since, for example, yeast which does express STI1 is lacking steroid receptors. It is possible that the heterocomplex may assist in the trafficking of a variety of proteins through the cytoplasm (37) or may act as a chaperone complex (10). STI1 appears to be transiently associated with this complex and could either be involved in regulating the proper functioning of the complex or merely be using the complex as a transport vehicle. It is noteworthy that all of the STI1-associated proteins described so far, including steroid receptors, HSC 70 and HSP 90, shuttle between cytosol and nucleus or have at least been implicated in the shuttling process (37). Shuttling between the two compartments is ascribed to the presence of NLS motifs as they are known or have been suggested for steroid receptors (38) or HSC 70 (39), respectively. Participation in the shuttling process of some nuclear proteins is believed to be due to the in vitro ability of HSC 70 to bind NLS sequences (40) or is based on an unknown mechanism in the case of HSP 90, as suggested by in vivo experiments (41). Our immunofluorescence data which stress the existence of a cytotoxic STI1 population besides the nuclear one together with the finding of a potential NLS within the mSTI1 amino acid sequence thus suggest that protein STI1 might be able to shuttle between the cytosolic and the nuclear compartment as well.

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Note Added in Proof—After submission of this manuscript, Chen et al. described similar interactions of hSTI1 with HSP70 and HSP90 (Chen, S., Prapapanich, V., Rimerman, R. A., Honore, B., and Smith, D. F. (1996) Mol. Endocrinol. 10, 682–693).

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