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Stch encodes the 'ATPase core' of a microsomal stress70 protein

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The stress70 protein chaperone family plays a central role in the processing of cytosolic and secretory proteins. We have cloned a human cDNA, designated *Stch*, that is conserved in rat tissues and which encodes a novel microsome-associated member of the stress70 protein chaperone family. *Stch* mRNA is constitutively expressed in all human cell types and is induced by incubation with the calcium ionophore A23187, but not by exposure to heat shock. Inspection of the predicted amino acid sequence reveals that the STCH product contains a unique hydrophobic leader sequence and shares homology within the amino terminal domains of the stress70 gene family, but has a 50 residue insertion within the ATP-binding domains and truncates the carboxyl terminal peptide-binding region. Immunofluorescent and subcellular analyses show that STCH migrates predominantly as a 60 kDa species and is enriched in a membrane-bound microsome fraction. In contrast to purified BiP and dnaK, however, STCH demonstrates ATPase activity that is independent of peptide stimulation. *Stch*, therefore, encodes a calcium-inducible, microsome-associated ATPase activity with properties similar to a proteolytically cleaved N-terminal HSC70/BiP fragment. This truncated stress70 molecule may allow increased diversity in cellular responses to protein processing requirements.

Key words: ATPase/cDNA cloning/microsome/protein chaperone/stress70

Introduction

The co-ordinated events underlying protein folding, translocation across membranes, post-translational modifications and multi-unit assembly are essential to the viability of all cellular organisms. Recent data have demonstrated that conserved members of the 'stress' or 'heat shock' protein (HSP) family participate in these processing events by their ability to bind denatured or misfolded peptide sequences and then to release these polypeptide chains by an ATP-dependent mechanism (reviewed in Pelham, 1986; Hightower, 1991; Gething and Sambrook, 1992). Although HSP members were first identified by their accumulation following cell exposure to elevated temperatures (Ritossa, 1962; Tissières *et al.*, 1974), it was later recognized that a significant subset of these proteins was constitutively expressed and that cell incubations with a variety of other metabolic poisons could result in gene induction (Welch *et al.*, 1983). In eukaryotes, certain HSP products have been localized to specific cellular fractions, such as the cytosol, nucleus, endoplasmic reticulum or mitochondria, and recent experimental models have implicated these 'chaperones' with facilitating protein transport across these specialized compartments (Chirico *et al.*, 1988; Deshaies *et al.*, 1988).

The stress70 gene family is complex and members from varied stages of evolution, including bacterial (*dnaK*) (Bardwell and Craig, 1984), yeast (*kar2*, *ssa1*, *ssa2* and *ssc1*) (Ingolia *et al.*, 1982; Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Normington *et al.*, 1989; Rose *et al.*, 1989) and mammalian species (*hsp70*, *hsc70*, *grp78/BiP* and *pbp74*) (Mues *et al.*, 1986; Munro and Pelham, 1986; Gething and Sambrook, 1992; Domanico *et al.*, 1993) have been studied. Structural analyses of these gene products have shown a highly conserved amino terminal domain that encodes ATP-binding and hydrolysis activity, and a less conserved carboxyl terminal portion that is required for peptide binding (Chappell *et al.*, 1987; Flajnik *et al.*, 1991; Rippmann *et al.*, 1991). The recognition that an *hsp70*-related gene encoded an abundant endoplasmic reticulum (ER) product identical to both the mammalian immunoglobulin binding protein (BiP) and the glucose-regulated protein (GRP78) suggested a specific role for stress70 proteins in the folding and assembly of newly synthesized proteins in the ER (Munro and Pelham, 1986). This confirmed the hypothesis that stress70 molecules participate in protein processing during normal and stressed conditions (Bole *et al.*, 1986; Gething *et al.*, 1986; Pelham, 1986). GRP78/BiP has since been widely studied and shown to be targeted and retained in the ER by an amino terminal signal peptide and a carboxyl terminal ER retention signal (the tetrapeptide KDEL) (Munro and Pelham, 1986, 1987). Functional experiments have demonstrated that GRP78/BiP can reversibly bind *in vitro* to short peptides with relatively hydrophobic amino acid residues that are proposed to represent exposed domains on unfolded or misfolded proteins within the ER. In addition, GRP78/BiP encodes a peptide-stimulated ATPase activity that may then drive the protein folding process toward completion (Flynn *et al.*, 1989, 1991).

We describe here the cloning of a constitutively expressed member of the stress70 protein chaperone family, designated *Stch*. Although *Stch* encodes a protein with striking amino acid identity to HSP70 and BiP, it differs from previously reported stress70 products by the presence of a unique hydrophobic signal sequence, a 50 amino acid insertion within the ATP-binding domain, and by the absence of the carboxyl terminal peptide-binding domain. These structural features predict the presence of a truncated stress70 product within protein secretory pathways that resembles the N-terminal proteolytic fragments of HSC70 and BiP

(Chappell *et al.*, 1987; Kassenbrock and Kelly, 1989). Using α-STCH antisera, we have confirmed these predictions and demonstrated that *Stch* encodes a smaller product (p60) which is highly enriched within the lumen of the cellular microsome fraction. In addition, we have shown that STCH exhibits ATPase activity that, in contrast to other HSP70-like molecules, is independent of peptide stimulation.

Results

Molecular cloning of a novel member of the stress70 protein chaperone family

In the course of experiments to study gene products that bind to the retinoblastoma protein, we isolated a cDNA clone from a K562 erythroleukemia cell line library that, on the basis of its predicted amino acid sequence, encoded a novel member of the stress70 protein chaperone family. This cDNA did not exhibit significant nucleotide homology to our initial probe, the retinoblastoma binding protein-1 gene (Otterson *et al.*, 1993), and we therefore concluded that it was serendipitously isolated from this cDNA library. We subsequently obtained overlapping cDNA clones and determined the nucleotide sequence of a 2.2 kilobase (kb) cDNA transcript that encoded a single long open reading frame of 471 codons, initiated by a consensus AUG flanking sequence (Kozak, 1984) (Figure 1). Analysis of the translated amino acid sequence using the BlastP protein homology search program (Altschul *et al.*, 1990) identified significant homology with the stress70 protein family clustered primarily in five ATP-binding domains (Bork *et al.*, 1992) (Figure 2). The amino acid identity in these domains defines this group as distinct from other less conserved ATP-binding proteins. Therefore, we have designated this gene as *Stch* (for *Stress/ch*aperone) and its encoded protein as STCH. In contrast to HSP70, STCH contains a unique hydrophobic leader peptide sequence, has a 50 residue insertion between the phosphate2 and adenosine ATP-binding sequence motifs (Bork *et al.*, 1992), and has truncated 236 and 227 carboxyl terminal residues present in HSP70 and BiP, respectively. We subsequently confirmed the nucleotide sequence of *Stch* by isolating and sequencing cDNA clones from an additional human cDNA library (H69, Stratagene) and by polymerase chain reaction (PCR)–amplification of first-strand cDNA (RT-PCR) from total RNA extracted from H2172 and H2009 human carcinoma cell lines.

Stch is conserved in human and rat tissues as a single-copy gene

To examine whether *Stch* exists as a single-copy gene, and whether it is conserved across evolution, we performed DNA blot analysis using an internal cDNA probe, designated

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GGTACAGTCATCACAAGCCTGTTCGGCGGGACTGTGATGGCCAGAGAGATGACGATCTTAGGATCGGCTGTTTTGACTCTCCTGTTGGCCGGCTATTTGGCACAACAGTATTTACCATTG 120
                        M  A  R  E  M  T  I  L  G  S  A  V  L  T  L  L  L  A  G  Y  L  A  Q  Q  Y  L  P  L

CCTACTCCTAAAGTGATTGGTATTGATCTTGGCACCACCTATTGTTCTGTTGGGGTGTTTTTTCCTGGCACAGGAAAAGTAAAGGTGATTCCAGATGAAAATGGGCATATCAGCATACCC 240
 P  T  P  K  V  I  G  I  D  L  G  T  T  Y  C  S  V  G  V  F  F  P  G  T  G  K  V  K  V  I  P  D  E  N  G  H  I  S  I  P

AGCATGGTGTCTTTTACTGACAATGATGTATATGTGGGATATGAAAGCGTAGAGCTGGCAGATTCAAATCCTCAAAACACAATATATGATGCCAAAAGATTCATAGGCAAGATTTTTACC 360
 S  M  V  S  F  T  D  N  D  V  Y  V  G  Y  E  S  V  E  L  A  D  S  N  P  Q  N  T  I  Y  D  A  K  R  F  I  G  K  I  F  T

GCAGAAGAGTTGGAGGCTGAAATTGGCAGATACCCATTTAAGGTTTTAAACAAAAATGGAATGGTTGAGTTTTCTGTGACAAGTAATGAGACCATCACAGTGTCCCCAGAATATGTTGGC 480
 A  E  E  L  E  A  E  I  G  R  Y  P  F  K  V  L  N  K  N  G  M  V  E  F  S  V  T  S  N  E  T  I  T  V  S  P  E  Y  V  G

TCTCGACTATTGTTGAAGTTAAAGGAAATGGCAGAGGCATATCTTGGAATGCCAGTTGCCAATGCTGTCATTTCTGTACCAGCAGAATTTGATCTAAAACAGAGAAATTCAACAATTGAA 600
 S  R  L  L  L  K  L  K  E  M  A  E  A  Y  L  G  M  P  V  A  N  A  V  I  S  V  P  A  E  F  D  L  K  Q  R  N  S  T  I  E

GCTGCTAACCTTGCAGGACTGAAGATTTTGAGGGTAATAAATGAACCCACAGCAGCAGCTATGGCCTATGGTCTCCACAAGGCTGACGTCTTCCACGTCTTGGTGATAGACTTGGGCGGA 720
 A  A  N  L  A  G  L  K  I  L  R  V  I  N  E  P  T  A  A  A  M  A  Y  G  L  H  K  A  D  V  F  H  V  L  V  I  D  L  G  G

GGAACTCTAGATGTGTCTTTACTGAATAAACAAGGAGGGATGTTTCTAACCCGAGCAATGTCTGGAAACAATAAACTTGGAGGACAGGACTTCAATCAGAGATTGCTTCAGTACTTATAT 840
 G  T  L  D  V  S  L  L  N  K  Q  G  G  M  F  L  T  R  A  M  S  G  N  N  K  L  G  G  Q  D  F  N  Q  R  L  L  Q  Y  L  Y

AAACAGATCTATCAAACATATGGCTTCGTGCCCTCTAGGAAAGAGGAAATCCACAGATTGAGACAAGCTGTGGAAATGGTCAAATTAAATCTGACTCTTCATCAATCTGCTCAGTTGTCA 960
 K  Q  I  Y  Q  T  Y  G  F  V  P  S  R  K  E  E  I  H  R  L  R  Q  A  V  E  M  V  K  L  N  L  T  L  H  Q  S  A  Q  L  S

GTATTACTAACGGTGGAGGAGCAGGACAGGAAGGAACCTCACAGTAGTGACACTGAACTGCCAAAAGACAAACTTTCCTCAGCAGATGACCATCGCGTGAACAGTGGGTTTGGACGTGGC 1080
 V  L  L  T  V  E  E  Q  D  R  K  E  P  H  S  S  D  T  E  L  P  K  D  K  L  S  S  A  D  D  H  R  V  N  S  G  F  G  R  G

CTTTCTGATAAGAAAAGTGGAGAAAGTCAGGTTTTATTTGAAACAGAAATATCACGGAAACTCTTTGATACCCTTAATGAAGACCTCTTTCAGAAAATACTGGTACCCATTCAGCAAGTA 1200
 L  S  D  K  K  S  G  E  S  Q  V  L  F  E  T  E  I  S  R  K  L  F  D  T  L  N  E  D  L  F  Q  K  I  L  V  P  I  Q  Q  V

TTGAAAGAAGGCCACCTGGAAAAGACTGAGATTGATGAGGTGGTTTTAGTTGGGGGYTCCACTCGTATTCCTCGGATCCGTCAAGTCATTCAAGAGTTCTTTGGAAAAGATCCCAACACA 1320
 L  K  E  G  H  L  E  K  T  E  I  D  E  V  V  L  V  G  G  S  T  R  I  P  R  I  R  Q  V  I  Q  E  F  F  G  K  D  P  N  T

TCTGTAGACCCTGACCTAGCAGTAGTAACGGGAGTGGCTATCCAAGCAGGGATTGATGGAGGCTCTTGGCCTCTCCAAGTCAGTGCTTTAGAAATTCCCAATAAGCATTTACAAAAAACC 1440
 S  V  D  P  D  L  A  V  V  T  G  V  A  I  Q  A  G  I  D  G  G  S  W  P  L  Q  V  S  A  L  E  I  P  N  K  H  L  Q  K  T

AACTTCAACTGAATTCTGCAGAAATAATGGTTATTTGTGAACTTGTCTGATGATCTCTTCCCATTTATCAGATTACCTTTTCCACAAAAGAAAGTCTCTAAAATATCACAGATTTACCTA 1560
 N  F  N  *

GAGGGCAACATTTAGATACAGGAAAATTTTACATAGTGTTTTGTCTTAGGATTAGACGTGACCAGATTGATCCTGTTTGATTTTGGAGAGATCCTATTCTAACAAATACTCTAAAATGAT 1680

AAAATTGAGGTACAACTCTCTTAAAAGAGTATGGATAACTATATTTTCTGGATTCTGGAGGTTGATAACCATATGCACTTAACATATATTCTATAAACATTAAGTAGTGCCAGTTATGAG 1800

ATTCCCAGTTCTTACTAAATTGTATTAGCAGGAGCTGGTAATTACTTGTATTATCACATGTAACTAATAATTTGAACTATACTTGAAGGACCGTGTTGATGTCAGGTATTTACAGTGGTT 1920

GGAAGATAGCAGTATTATTAGATAAGCTGCATACGTAATATTCAGTAACTGCCATATTATATAACAAATTTACATTCACAAATTCAGTATCCTGTTAAGTGTCATATTCTTGTAATCTGC 2040

ATTCTCCAGGAGTTTTATGTGTTTAATAGATGAATTTATTTTATTTCTAAAGGTATTCAAATGTTTCAGCACCATATAATAGAAATACCCAATTATATTCTAGTTCCTTTATGTCCTGTA 2160

CATCATTCTCTGCTTGGATTTCCATTATTCTGTTTGGTTAGAGAATAAAATTGGTAATTGCATTTGAAAAAAAAAAAAAAAAAAA                                    2246
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Fig. 1. Nucleotide and translated amino acid sequence for the human *Stch* cDNA. Four potential *N*-glycosylation sites (N-X-S/T) at codons 137, 184, 298 and 427 are shown in bold. The two peptide domains used to raise polyclonal antisera are underlined. Amino acid residues are designated by their single-letter code. The GenBank/EMBL accession number for the sequences presented in this paper is UO4735.

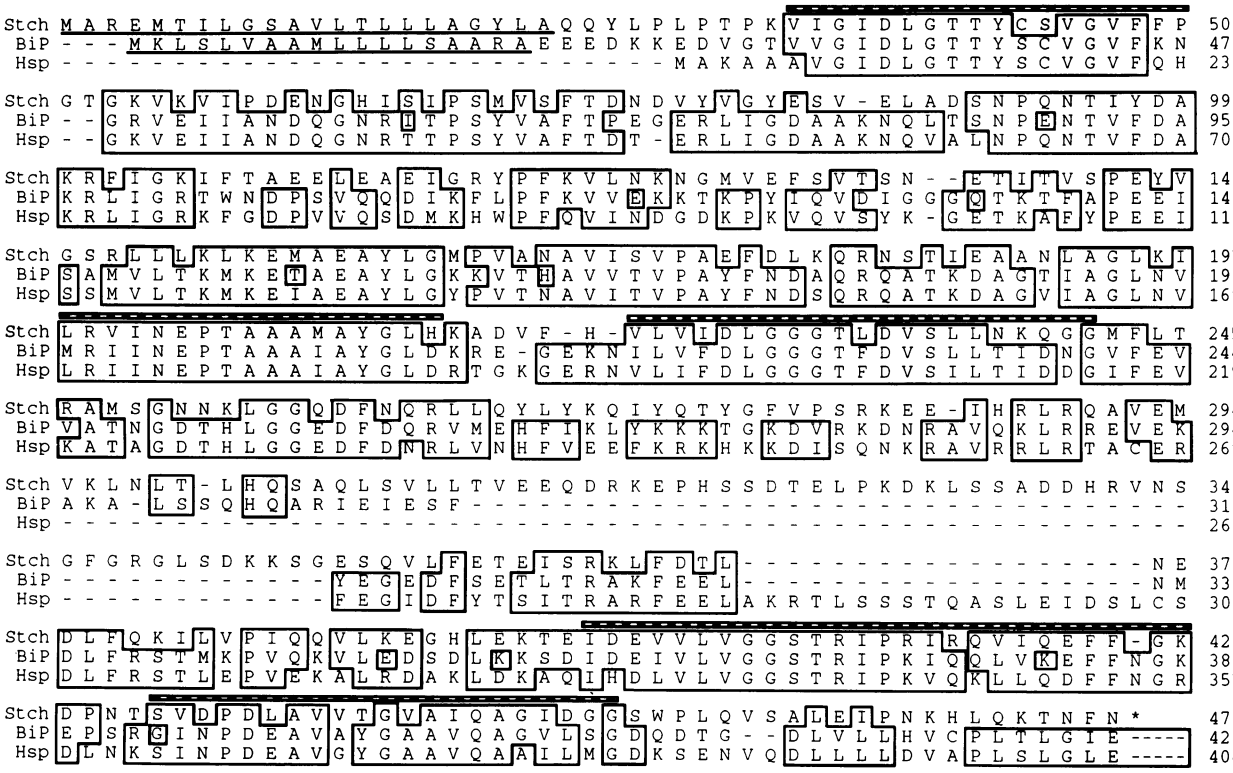

Fig. 2. Amino acid alignment of STCH with the human HSP70 (Hunt and Morimoto, 1985) and the human BiP (Ting and Lee, 1988) products. Regions of amino acid homology are boxed, including the conservative substitutions V/I/L/M, S/T, E/D, Y/F and K/R. Hydrophobic leader sequences for STCH and BiP are underlined. Five conserved regions involved in ATP binding (phosphate1, connect1, phosphate2, adenosine and connect2) (Bork *et al.*, 1992) are shown sequentially by a striped box above the sequence. The carboxyl terminal domains of BiP (227 codons) and HSP70 (236 codons) are not shown.

pStch1. Genomic DNA from human, rat and *Drosophila* tissues was digested to completion with *Bam*HI and *Eco*RI restriction enzymes, and Southern blot analysis was performed under low-stringency hybridization and washing conditions (Figure 3). This analysis demonstrated that *Stch* is present as a single locus in human and rat, but is not detectable under these conditions in the *Drosophila* genome. In addition, this experiment shows that the *Stch* cDNA does not hybridize at low stringency with other members of the stress70 gene family.

Stch is constitutively expressed in all tissues and is not inducible by heat shock

We next examined the mRNA pattern of *Stch* and observed that it is expressed as 4.4 and 2.4 kb transcripts in all human adult tissues examined, although the steady-state levels of the 2.4 kb transcript varied greatly (Figure 4A). To determine if *Stch* expression is temperature inducible or constitutive, we evaluated mRNA levels following heat shock. In contrast to HSP70 (Figure 4B, lanes 5 and 6), *Stch* is not inducible by heat shock (Figure 4B, lanes 3 and 4); however, steady-state levels were enhanced following 24 h treatment of cells with the calcium ionophore A23187 (Figure 4B, lanes 1 and 2). These results resemble the pattern of induction observed with GRP78/BiP (Munro and Pelham, 1986) and characterize *Stch* as a constitutively expressed member of the stress70 family. Although the molecular basis for the two *Stch* transcripts is unknown, we demonstrated that non-overlapping cDNA probes from the 5′ (nucleotide co-ordinates # 57–413), middle (co-ordinates # 364–1155) and 3′ untranslated (co-ordinates # 1533–2025) portions of human *Stch* detected both 4.4 and 2.4 kb transcripts in RNA extracted from human tumor cell lines (data not shown), suggesting that both transcripts encode *Stch* specific sequences.

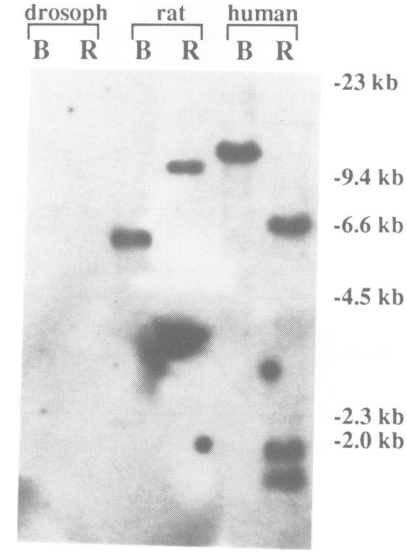

Fig. 3. DNA analysis of *Stch* demonstrating conservation in rodent and human genomes. A partial cDNA corresponding to *Stch* nucleotide co-ordinates # 364–1155 (pStch1) was used as a probe under lowered stringency hybridization and washing conditions described in Materials and methods.

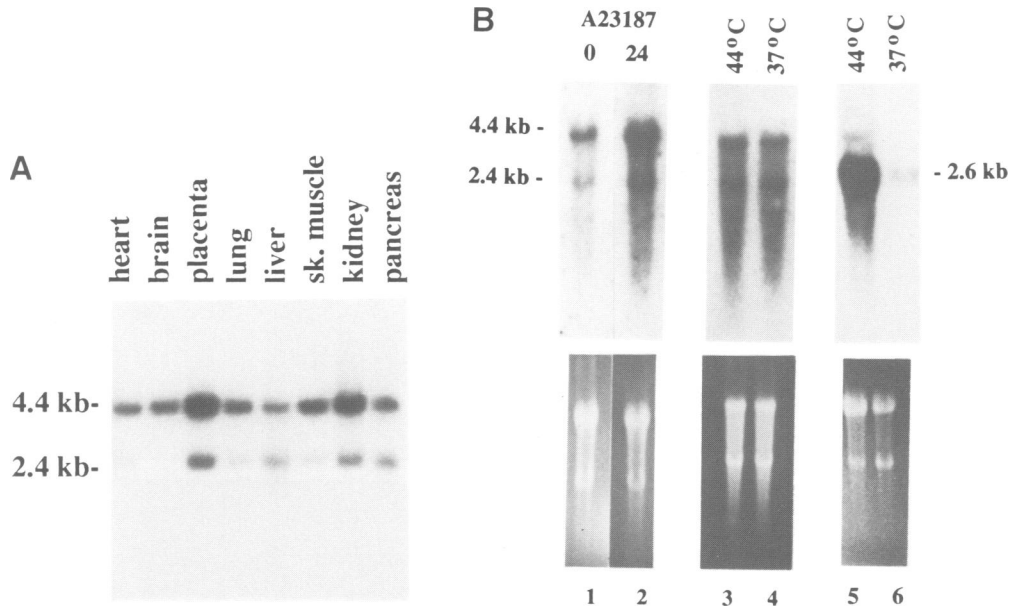

Fig. 4. (**A**) *Stch* mRNA expression in selected adult human tissues using the pStch1 probe. Hybridization and washing conditions are as described in Materials and methods. (**B**) Effect of heat shock and A23187 exposure on steady-state *Stch* (lanes 1–4) and *Hsp70* (lanes 5 and 6) mRNA expression. Ten micrograms of total cellular RNA were harvested from H2172 cells under control conditions (lanes 1, 4 and 6) or after exposure to 24 h of 7 μM A23187 (lane 2) or 2 h of 44°C (lanes 3 and 5). The rRNA pattern of the same gel is shown after ethidium bromide staining. pStch1 and pH2.3 (Hunt and Morimoto, 1985) were used as *Stch* and *Hsp70* cDNA probes, respectively.

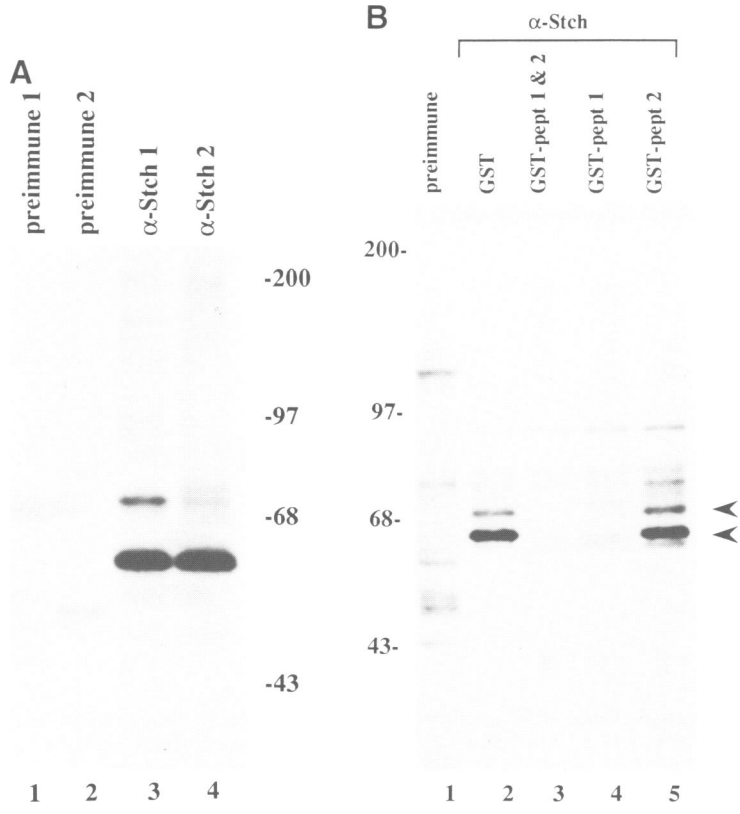

Fig. 5. (**A**) Immunoprecipitation of STCH from [^{35}S]methionine-labeled cell lysates. H2172 cells were metabolically labeled with [^{35}S]methionine and immunoprecipitated with either pre-immune sera (lanes 1 and 2) or α-STCH antisera (lanes 3 and 4) obtained from two different rabbits. (**B**) Immunoprecipitation from [^{35}S]methionine-labeled cell lysates using STCH antisera pre-adsorbed with different immunizing peptides. Lane 1: pre-immune sera; lane 2: α-STCH antisera pre-adsorbed with glutathione–Sepharose linked to GST alone; lane 3: antisera pre-adsorbed with GST–STCH peptides 1 and 2; lane 4: antisera pre-adsorbed with GST–peptide 1 alone; lane 5: antisera pre-adsorbed with GST–peptide 2 alone.

Immunoprecipitation of the human STCH product

To address the functional properties of this new protein product, we raised polyclonal α-STCH antisera directed against glutathione S-transferase (GST)–STCH fusion proteins that encoded two distinct STCH domains (Figure 1, peptide 1: codons 105–156 and peptide 2: codons 261–395)

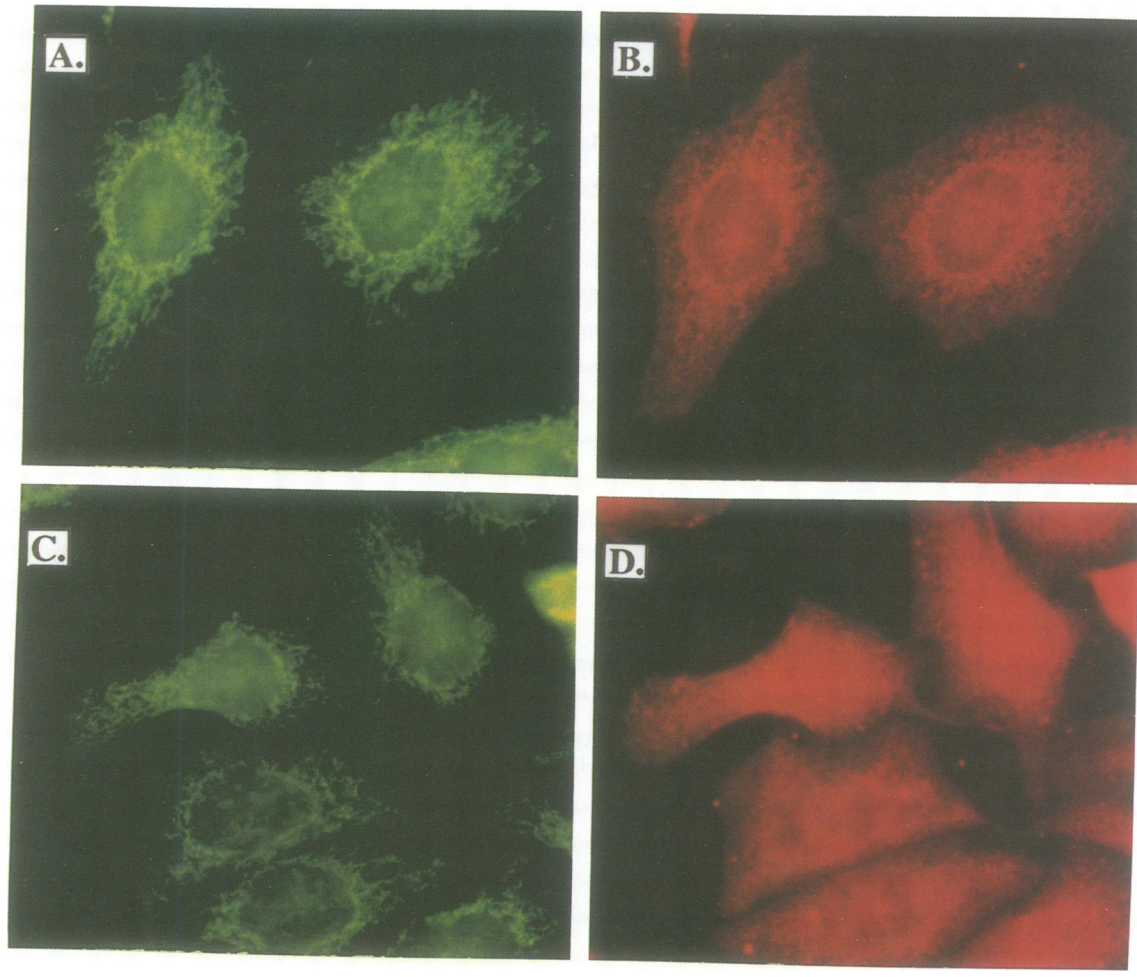

Fig. 6. Immunofluorescent analysis. HeLa cells were double labeled with α-STCH and α-BiP using fluorescein and Texas Red, respectively (**panels A** and **B**) or with α-STCH and α-HSP70 using fluorescein and Texas Red, respectively (**panels C** and **D**). Magnification 630×.

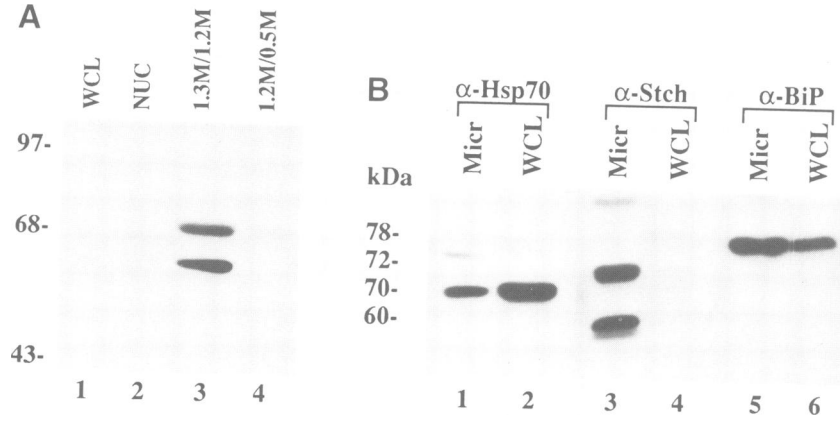

Fig. 7. (**A**) Subcellular fractionation assay. A total of 50 μg of protein from K562 whole-cell lysates (WCL), nuclear pellet (NUC), the 1.3 M sucrose/1.2 M sucrose interface pellets (1.3 M/1.2 M), or the 1.2 M sucrose/0.5 M sucrose interface pellets (1.2 M/0.5 M) (lanes 1–4, respectively) were subjected to immunoblotting with the α-STCH antisera as described in Materials and methods. (**B**) Immunoblot analysis comparing 50 μg of protein from K562 whole-cell lysates (WCL) or microsome preparations (Micr) for STCH, HSP70 and BiP protein expression.

with restricted homology to HSP70 and BiP. The antisera were pre-adsorbed over glutathione–Sepharose beads (Pharmacia, Piscataway, NJ) linked to the GST leader peptide prior to subsequent experiments in order to reduce non-specific reactivity. We used the pre-cleared α-STCH antisera from two different rabbits to immunoprecipitate [^{35}S]methionine-labeled H2172 cell lysates and demonstrated that STCH migrates on SDS–PAGE predominantly as a 60 kDa species, although a 72 kDa band was also observed (Figure 5A, lanes 3 and 4). In addition, antisera from both rabbits gave similar results, while pre-immune sera were associated only with non-specific bands (Figure 5A, lanes 1 and 2).

We repeated the immunoprecipitation using α-STCH

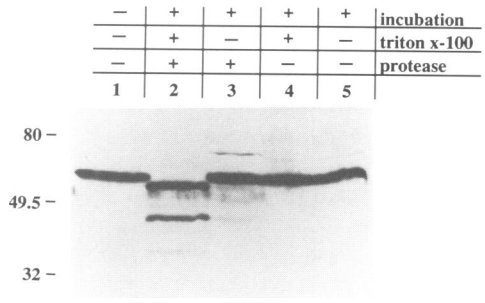

Fig. 8. Protease digestion of microsome preparations in the presence or absence of Triton X-100. Incubation conditions are depicted as described in Materials and methods.

antisera from rabbit 2 to test for specificity to the immunizing peptides 1 and 2 (Figure 5B). In this experiment, we pre-adsorbed the antisera over glutathione−Sepharose beads linked to the GST leader sequence (lane 2), GST−peptide 1 and 2 (lane 3), GST−peptide 1 (lane 4), or GST−peptide 2 (lane 5). We observed that peptide 1, but not peptide 2 or the GST leader peptide, could compete for specific binding to the 60 and 72 kDa bands. This experiment confirms the specificity of the α-STCH antisera for the STCH peptide 1 domain and for the 60 and 72 kDa products. The p72 species seen on immunoprecipitation studies is significantly larger than that predicted by the *Stch* cDNA and, although HSP70 and BiP are not recognized by our antisera (see below), p72 may represent another HSP70-related protein that contains an epitope similar to STCH peptide 1. Another possibility is that p72 represents the product of an alternatively spliced *Stch* transcript; however, we have been unable to isolate a *Stch* cDNA with evidence for an alternative open reading frame.

STCH is microsome associated

Double-labeling immunofluorescence was performed on preparations of HeLa cells utilizing α-STCH antisera (pre-adsorbed over the GST leader peptide) tagged with fluorescein and either α-BiP or α-HSP70 tagged with Texas Red. These experiments localized STCH to the cellular cytoplasm in a pattern that resembled, but was not identical to, the ER staining of BiP (Figure 6A and B), and was distinct from the HSP70 (Figure 6C and D) staining pattern. Specificity of the α-STCH antisera was again confirmed by absent cytoplasmic staining with antisera pre-adsorbed with STCH peptide 1 (data not shown).

To further define the subcellular localization of STCH, microsome preparations from 5×10^8 K562 cells were prepared by centrifugation of cell homogenates over non-continuous sucrose gradients as previously described (Tartakoff and Vassalli, 1979). We subjected 50 μg of protein from the subcellular fractions as well as 50 μg of whole cell protein (representing $\sim 10^6$ cells) to an immunoblot analysis with the α-STCH antisera. The results of this fractionation demonstrate that STCH is enriched in the denser microsome fraction isolated at the 1.3 M/1.2 M sucrose interface (Figure 7A, lane 3). In contrast, STCH levels are significantly reduced in the washed nuclear pellet and in the lighter microsome fraction harvested at the 1.2 M/0.5 M sucrose interface (Figure 7A, lanes 2 and 4). These experiments also show that the α-STCH antisera react with the 72 kDa species in immunoblot assays and that p72 fractionates with p60.

We repeated the subcellular fractionation and subjected equal amounts of the whole-cell protein lysate or the denser microsome fraction to immunoblot analysis in triplicate (Figure 7B). We divided the nitrocellulose filter and probed with either α-HSP70 (lanes 1 and 2), α-STCH (lanes 3 and 4) or α-BiP (lanes 5 and 6). This experiment demonstrates that the 60 and 72 kDa species recognized by the α-STCH antisera do not co-migrate or cross-react with either human HSP70 or human BiP, and confirms that, in contrast to the cytosolic HSP70 protein, STCH is enriched in the microsome fraction. We also observed that STCH appeared to be more highly enriched in the microsome fraction than BiP, suggesting either that BiP may be released during the microsome preparation or that both proteins are localized in different subcellular compartments.

To distinguish whether STCH is peripherally associated with the outer membrane or localized to the lumen of microsomes, we conducted a protease digestion experiment in the presence or absence of detergent (Triton X-100). We observed that the STCH product obtained from the dense microsome fraction of a discontinuous sucrose gradient (as described above) was susceptible to protease digestion only after solubilization of the membranes with mild detergent (Figure 8), consistent with an intraorganellar localization of the STCH product.

STCH demonstrates peptide-independent ATPase activity

Inspection of the primary amino acid sequence of the STCH product predicts a functional ATPase molecule, and to test for enzyme activity we constructed a GST−*Stch* plasmid that encoded a fusion protein including STCH codons 4−471. To exclude contaminating sources of ATPase, the GST leader peptide and the GST−STCH affinity-purified fusion proteins were eluted off glutathione−Sepharose beads, then purified by anion-exchange chromatography (Mono Q) and the protein composition of the column fractions was analyzed by SDS−PAGE (Figure 9A). We observed that the peak of ATPase activity of the GST−STCH product coincided with fractions 11 and 12, while no measurable ATPase activity was detected in these fractions from the GST leader sequence (data not shown). We pooled fractions 11 and 12, measured ATPase activity in the presence or absence of two different peptides and compared these data with purified BiP and with the bacterial HSP70, dnaK (Figure 9B). As predicted from the structural analysis showing a truncated carboxyl terminus, STCH did not exhibit a peptide-stimulated enhancement of ATPase activity, in contrast to BiP (Flynn *et al.*, 1989) and dnaK. We also observed that the K_m for the unstimulated STCH ATPase was 2−3 μM ATP, and the measured ATPase activity was linear with respect to time and concentration over the range tested (data not shown).

Discussion

We have identified a human cDNA that encodes a novel member of the stress70 protein chaperone family. Analysis of the STCH product reveals several features that resemble the ER lumenal protein BiP, including constitutive expression in human tissues examined and mRNA induction following cell incubation with the calcium ionophore A23187. This

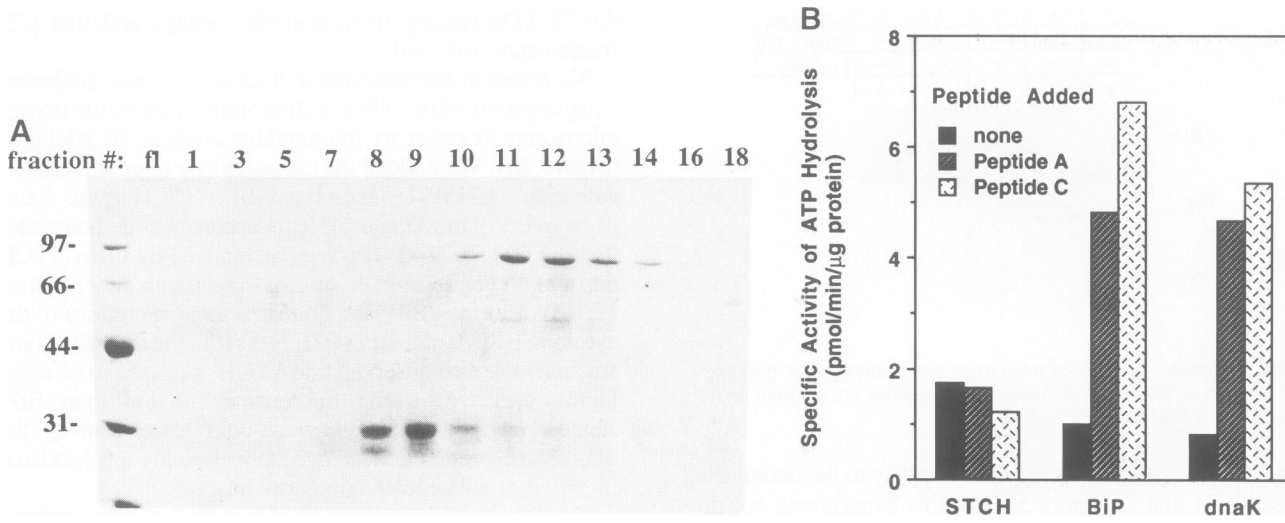

Fig. 9. (**A**) Anion-exchange chromatography of the affinity purified GST−STCH. The GST−STCH fusion protein was expressed and purified from bacteria using glutathione−Sepharose and then fractionated on a Mono Q column using a 0−500 mM NaCl gradient. Fractions 11 and 12 were pooled for further ATPase enzyme assays. (**B**) STCH exhibits peptide-independent ATPase activity. ATPase activity was measured on the pooled Mono Q fractions from GST−STCH or with purified bovine BiP and purified dnaK in the absence or presence of either peptide A or C under conditions described in Materials and methods.

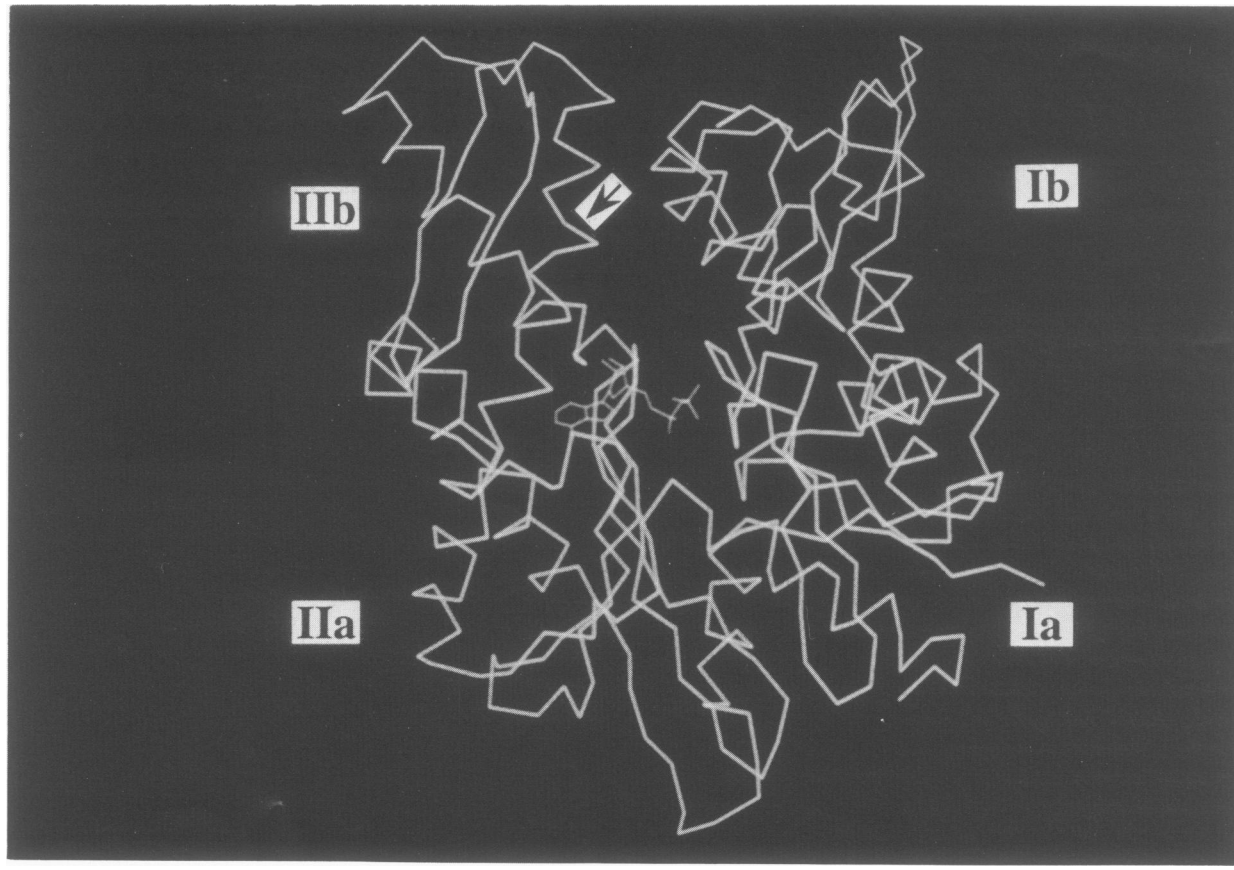

Fig. 10. Three-dimensional structure of the N-terminal fragment of HSC70 with the four protein-folding domains (Ia, Ib, IIa and IIb) is depicted [modified from Flaherty *et al.* (1990)] showing a bound ATP molecule (fine lines) and the approximate location of the amino acid insertion encoded on the STCH molecule (arrow).

pattern of expression suggests a functional role that may be responsive to calcium ion fluxes in a membrane-based compartment. Consistent with this prediction, we observed that STCH contains a 22 residue hydrophobic leader peptide that is analogous, but not identical, to the GRP78/BiP signal sequence, and subcellular localization and protease digestion assays demonstrated significant enrichment of the STCH product within the lumen of microsome membranes. In contrast to BiP, however, STCH does not encode a consensus ER retention signal (Munro and Pelham, 1987) and double labeling immunofluorescent analysis reveals that STCH resembles, but is not identical to, the BiP staining.

Surprisingly, inspection of the STCH amino acid sequence reveals a protein that is predicted to encode a 'core ATPase' molecule that has been observed previously only after the proteolytic cleavage of the HSC70 or BiP products *in vitro* (Chappell *et al.*, 1987; Kassenbrock and Kelly, 1989). STCH has ~33% identity and 43% homology to both human HSP70 and human BiP, localized primarily to the five ATP-binding/hydrolysis consensus domains (Bork *et al.*, 1992) and their immediate flanking regions (Figure 2). In addition, however, STCH contains a 50 residue insertion within the ATP-binding domain between the phosphate2 and adenosine ATP-binding sequence motifs (Flaherty *et al.*, 1990; Bork *et al.*, 1992), and has truncated the carboxyl terminal peptide-binding sequences just downstream of the last consensus ATP-binding domain. This resembles the N-terminal proteolytic digestion products from both HSC70 and BiP that include a transient 60 kDa species followed by the accumulation of a 44 kDa N-terminal fragment (Chappell *et al.*, 1987; Kassenbrock and Kelly, 1989). Of interest, STCH terminates two residues downstream of a hypothetical cleavage site for the 44 kDa N-terminal fragment of HSC70 (Chappell *et al.*, 1987). *In vivo* immunoprecipitation, however, detects a larger 60 kDa STCH protein. If STCH is the structural and functional (see below) analog of the 44 kDa fragment, the discrepancy in size may be partially explained by conformational changes induced by the amino acid insertion within the STCH molecule, particularly since the fusion GST−STCH molecule also migrates more slowly than predicted by the primary amino acid sequence (Figure 9A). In addition to p60, we also detected a p72 species that was specific for the STCH peptide 1 epitope. Although it remains possible that the p72 species may represent the product of an alternatively spliced *Stch* mRNA, we have been unable to isolate such a transcript. Another possibility, however, is that p72 represents a distinct HSP70-like molecule that shares sufficient homology with STCH to allow reactivity with our antisera, although we note that neither BiP nor HSP70 showed cross-reactivity in our protein analyses.

Since the three-dimensional structure of the N-terminal fragment of HSC70 has been recently published, we determined the predicted location of the 50 residue insertion within the STCH molecule and observed that it was outside of the ATP-binding pocket within the HSC70 protein-folding domain, IIb (Figure 10). To examine directly whether ATPase activity would be affected by this insertion and by the absence of a carboxyl terminal domain, we assayed for enzyme activity using purified BiP, dnaK and a recombinant STCH product representing codons 4−471. We observed that, in contrast to BiP and dnaK, STCH exhibited ATPase activity that was independent of peptide stimulation. STCH, therefore, is functionally equivalent to the 'ATPase core' of bovine HSC70 which retained ATPase activity that was uncoupled from a dependence on clathrin binding (Chappell *et al.*, 1987). We have, therefore, identified an *in vivo* HSP70-related molecule with similar properties to the *in vitro* proteolytic HSC70 fragment, which suggests a unique role for STCH in protein processing, but also raises the question of how it regulates its ATPase activity and how it is retained in its intraorganellar compartment. The identification of a cell type with absent or defective STCH expression may help define its functional role, which may allow for increased diversity in the cellular response to protein processing requirements.

Materials and methods

Cell lines and propagation

K562 erythroleukemia cells and HeLa cells were obtained from the American Type Culture Collection (Rockville, MD), and the H2009 and H2172 cell lines were derived from patients with non-small cell lung cancer. All cell lines were propagated at 37°C/6% CO_2 in RPMI medium supplemented with 10% fetal calf serum.

cDNA cloning and sequencing

The initial *Stch* cDNA clone was obtained by screening a K562 λgt11 library with a [^{32}P]CTP random primed probe corresponding to the retinoblastoma binding protein-1 gene (Otterson *et al.*, 1993). Overlapping cDNA clones were subsequently obtained by screening a K562 λgt11 library and an H69 Uni-ZAP library (Stratagene, La Jolla, CA) with a [^{32}P]CTP random primed probe derived from the partial *Stch* cDNA clone (pStch1: nucleotide co-ordinates: # 364−1155). Hybridization and washing conditions for the library screenings were as previously described (Davis *et al.*, 1986), and positive cDNA inserts were subcloned into the pT7 vector (Novagen, Madison, WI) and subjected to nucleotide sequencing as described by the manufacturer (US Biochemical, Cleveland, OH). Additional 5′ sequence was obtained by anchored PCR techniques as described by the manufacturer (Life Technologies, Gaithersburg, MD). Nucleotide sequence was confirmed from at least two independent PCR products.

Nucleic acid analysis

Ten micrograms of genomic DNA from human, rat and *Drosophila* tissues were digested to completion with either *Bam*HI or *Eco*RI (Life Technologies), and subjected to Southern blot analysis as previously described (Davis *et al.*, 1986) using the ^{32}P-labeled pStch1 cDNA probe. Approximately 2 × 10^7 c.p.m. of radiolabeled probe were incubated overnight with the blotted nitrocellulose membrane in 10% dextran, 4 × SSC, 40% formamide, 1 × Denhardt's, 20 μg/ml herring sperm DNA, 20 mM Tris (pH 7.4) at 37°C. Filters were washed sequentially in 2 × SSC, 0.1% SDS and 0.1 × SSC, 0.1% SDS at 37°C, followed by autoradiography.

Two micrograms of poly(A) RNA extracted from human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas tissues were size fractionated on a formaldehyde/agarose gel and transferred to a nylon membrane (Clontech, Palo Alto, CA) which was hybridized overnight with the ^{32}P-labeled pStch1 probe at 42°C in the hybridization buffer described above and washed at 50°C in the wash solutions described above.

A total of 3 × 10^6 cells were subjected to heat shock (44°C for 2 h) or a 24 h exposure to 7 μM of the calcium ionophore A23187 (Calbiochem, San Diego, CA) and total RNA was harvested by guanidine isothiocyanate extraction (Chirgwin *et al.*, 1979). Ten micrograms of RNA from each sample were size fractionated on a formaldehyde/agarose gel and transferred to nitrocellulose, followed by hybridization with the ^{32}P-labeled pStch1 probe or the *Hsp70* cDNA probe, pH2.3 (Hunt and Morimoto, 1985), and autoradiography. Ethidium bromide staining of the 28S and 18S rRNA subunits was evaluated to confirm the amount and integrity of RNA loaded.

Generation of α-STCH antisera

To generate STCH-specific antisera, we produced recombinant STCH fusion proteins that encoded two regions (peptide 1: codons 105−156 and peptide 2: codons 261−395) that exhibited reduced homology to other previously reported stress70 proteins. Oligonucleotide pairs for peptide 1, sense: 5′-GGATCCGGCAAGATTTTTACCGCAGAAGAG-3′ and antisense: 5′-GGATCCTTACTTTAACTTCAACAATAGTCGAGA-3′; and for peptide 2, sense: 5′-GGATCCAGATTGCTTCAGTACTTATATAAA-CAG-3′ and antisense: 5′-GGATCCTTAGTGGCCTTCTTTCAATAC-TTGCTG-3′, were designed to subclone PCR products in-frame into the *Bam*HI site of the bacterial expression plasmid pGEX2T (Pharmacia). The nucleotide sequence of the fusion expression vectors was confirmed and GST−STCH fusion proteins were produced as previously described (Smith and Johnson, 1988). Approximately 1 mg of freshly purified fusion proteins (GST−STCH peptides 1 and 2) was mixed with an equal amount of Freund's adjuvant and injected i.m. into two New Zealand White rabbits three times over a 6 week period. Antisera were pre-adsorbed four times over glutathione−Sepharose attached to the GST leader peptide. Antisera were also pre-adsorbed over GST−STCH peptides 1 and 2 as indicated in Results.

Immunoprecipitation and immunoblot analyses

A total of 3 × 10^6 cells were washed and pre-incubated for 1 h in RPMI medium minus methionine (Gibco, Grand Island, NY) and then supplemented

with 100 μCi/ml [^{35}S]methionine (Amersham Life Sciences, Arlington Heights, IL) for 3 h at 37°C. Labeled cells were washed with phosphate-buffered saline (PBS) and lysed in buffer [50 mM Tris−HCl (pH 7.4), 250 mM NaCl, 50 mM NaF, 0.1% NP-40 supplemented with 1 mM phenylmethyl-sulfonylflouride (PMSF)]. The supernatant was clarified by centrifugation at 14 000 r.p.m. at 4°C for 5 min and used directly for immunoprecipitation. [^{35}S]Methionine-labeled cell lysates were incubated with either α-STCH antisera or pre-immune sera for 1 h at 4°C, followed by a 1 h incubation with washed protein A−Sepharose CL-4B (Pharmacia). The immune precipitates were washed five times in lysis buffer and subjected to SDS−PAGE and autoradiography.

For immunoblot analyses, unlabeled cells were lysed as described above and equal amounts of protein were size fractionated by 7.5% SDS−PAGE, transferred to nitrocellulose and incubated overnight with α-STCH antisera at a 1:200 dilution, or with α-BiP or α-HSP70 (StressGen, Victoria, BC) as directed by the manufacturer. Filters were washed and exposed to [^{125}I]protein A (Amersham) for 90 min, followed by autoradiography.

Immunofluorescence

Approximately 2.5 × 10^4 HeLa cells were split into 8-multichamber glass slides and allowed to grow for 24 h. The cells were fixed in 4% paraformaldehyde for 30 min, permeabilized in 0.5% Triton X-100 for 10 min and blocked for 30 min in 4% bovine serum albumin (BSA) in PBS (all subsequent dilutions were prepared in this solution). Polyclonal α-STCH antisera were incubated at a 1:10 dilution for 2 h, followed by a 30 min incubation with a 1:20 dilution of swine α-rabbit conjugated to fluorescein (Dako, Carpinteria, CA). Monoclonal antibodies to BiP and HSP70 (StressGen) were diluted 1:100 and incubated for 30 min, followed by a 30 min incubation with a 1:50 dilution of goat α-mouse conjugated to Texas Red (Oncogene Science, Uniondale, NY). All incubations were performed sequentially at room temperature and in the dark following the initial application of fluorescent conjugates. The specimens were examined with a Zeiss fluorescence microscope.

Subcellular fractionation

To prepare subcellular fractions for immunoblotting analysis, 5 × 10^8 K562 cells were resuspended in 10 mM Tris−HCl (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$ on ice for 10 min. Cell membranes were then disrupted by 15 strokes with a Dounce homogenizer and centrifuged at 1000 *g* for 5 min. The nuclear fraction (pellet) was washed in TKM [50 mM Tris−HCl (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$] and resuspended in lysis buffer. The supernatant was made isotonic with TKM and a 2× solution of 2.4 M sucrose in TKM was added for a final concentration of 1.2 M sucrose, as previously described (Tartakoff and Vassalli, 1979). We prepared a non-continuous sucrose gradient with successive layers of 2.0 M sucrose, 1.3 M sucrose, post-nuclear supernatant in 1.2 M sucrose, 0.5 M sucrose and TKM. The sucrose gradient was then centrifuged at 50 000 r.p.m. in an SW 50.1 rotor (Beckman, Palo Alto, CA) for 18 h. The 1.3 M sucrose/1.2 M sucrose interface and the 1.2 M sucrose/0.5 M sucrose interface were diluted to 5 ml in TKM and spun for 1 h at 50 000 r.p.m. in the SW 50.1 rotor. The pellets, representing a crude microsome preparation, were resuspended in lysis buffer and used for immunoblotting analyses as described above.

Protease digestion

Microsome membrane preparations from K562 cells were prepared as described above. The membrane aliquots (15 μl) were incubated for 5 min with or without 1% Triton X-100 prior to the addition of 0.3 μg proteinase K (Boehringer Mannheim). After 10 min at 30°C to allow protease digestion to occur, the reaction was stopped by the addition of 1 mM PMSF. Control reactions without added protease were also performed. Samples were analyzed by SDS−PAGE (10% acrylamide), followed by immunoblotting with the α-STCH antisera as described above.

ATPase activity

A GST leader peptide and a near full-length GST−STCH fusion protein, representing STCH codons 4−471, were purified from bacterial proteins using glutathione−Sepharose beads as described previously (Smith and Johnson, 1988). The proteins were released from the beads by incubation with 5 mM reduced glutathione in 50 mM Tris (pH 8.0), 1 mM DTT, for 10 min at 4°C. The proteins were separated from the resin by centrifugation and the elution was repeated. Eluted protein was then loaded onto an anion-exchange column (Mono Q, Pharmacia) and fractionated with a linear salt gradient [0−500 mM NaCl in 20 mM Tris (pH 7.5)]. The GST−STCH fusion protein eluted between 275 and 325 mM NaCl, while the GST eluted at a position corresponding to 175−220 mM NaCl. Pooled fractions were dialyzed against 500 vols of ATPase buffer [50 mM HEPES (pH 7.0), 25 mM KCl, 2 mM $MgSO_4$] and then concentrated by ultrafiltration (Centricon-10, Amicon Corp.). ATPase assays with the GST−STCH (0.8 μg) were performed as previously described (Flynn *et al.*, 1989) in the presence or absence of 1 mM peptide A (sequence: KRQIYTDLEMNRLGK) or 0.5 mM peptide C (sequence: KLIGVLSSLFRPK). For comparison, parallel incubations with bovine BiP (1.0 μg) and the bacterial dnaK (0.9 μg) were also performed. BiP (Flynn *et al.*, 1989) and dnaK (Mensa-Wilmot *et al.*, 1989) proteins were purified as previously described. Protein concentrations were determined using the BCA colorimetric analysis using BSA as a standard (Smith *et al.*, 1985).

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