A Mouse Homologue of the *Drosophila* Tumor Suppressor *l(2)tid* Gene Defines a Novel Ras GTPase-activating Protein (RasGAP)-binding Protein*

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p120 GTPase-activating protein (GAP) down-regulates Ras by stimulating GTP hydrolysis of active Ras. In addition to its association with Ras, GAP has been shown to bind to several tyrosine-phosphorylated proteins in cells stimulated by growth factors or expressing transforming tyrosine kinase variants. Here we report the cloning and characterization of a novel GAP-binding protein, mTid-1, a DnaJ chaperone protein that represents the murine homolog of the *Drosophila* tumor suppressor *l(2)tid* gene. Three alternatively spliced variants of mTid-1 were isolated, two of which correspond to the recently identified hTid-1, and hTid-1s forms of the human TIDI gene that exhibit opposing effects on apoptosis. We demonstrate that both cytoplasmic precursor and mitochondrial mature forms of mTid-1 associate with GAP in vivo. Interestingly, although mTid-1 is found tyrosine-phosphorylated in v-src-transformed fibroblast cells, GAP selectively binds to the unphosphorylated form of mTid-1. In immunofluorescence experiments, GAP and Tid-1 were shown to colocalize at perinuclear mitochondrial membranes in response to epidermal growth factor stimulation. These findings raise the possibility that Tid chaperone proteins may play a role in governing the conformation, activity, and/or subcellular distribution of GAP, thereby influencing its biochemical and biological activity within cells.

As a guanine nucleotide-binding protein, Ras cycles between an active GTP-bound and inactive GDP-bound conformation (1). Down-regulation of Ras activity is attributed to the cytosolic Ras GTPase-activating Protein (GAP)1 (2). GAP stimulates the weak intrinsic GTPase activity of Ras, accelerating the hydrolysis of bound GTP to GDP, thereby terminating mitogenic signals elicited by Ras proteins. In addition to its role as a negative regulator of Ras, it has been suggested that GAP may exert an effector function in the control of cytoskeletal reorganization and cell migration (3, 4). Although Ras binding and catalytic activity of GAP reside in its C terminus, the N-terminal sequences consist of a number of conserved protein modules including src-homology (SH) 2 and 3 protein interaction domains that facilitate the formation of signaling complexes, which may couple GAP to upstream regulators of Ras or downstream effector targets (5). Indeed, activated tyrosine kinases that phosphorylate GAP also promote its association via its SH2 domains, with several phosphorytose-containing proteins, including members of the p62\(^{2\text{ub}}\) family of proteins (6–12) and Rho/Rac GTPase-activating protein, p190 (13, 14). p62\(^{2\text{ub}}\) codes for a pleckstrin homology-containing adaptor protein first noted as one of the most prominently tyrosine-phosphorylated proteins in v-src-, v-fps-, and v-abl-transformed cells. Moreover, its hyperphosphorylation levels correlate with the transformation phenotype of these oncogenic products (7, 15). These observations led to the hypothesis that at least some of the transforming capabilities of oncogenic tyrosine kinases might be conferred by p62\(^{2\text{ub}}\) by virtue of its constitutive occupancy of the SH2 domains of GAP, which may have negative regulatory effects on GAP activity. Consistent with this notion, the binding of phosphorylated p62\(^{2\text{ub}}\) to GAP has been shown to significantly impair the GTPase-promoting activity of GAP towards Ras in vitro (16). By contrast, p190 functions as a Rho/Rac GTPase-activating protein and may serve as a RasGAP effector, providing an interface between the Ras-signaling pathway and proteins of the Rho/Rac pathway that regulate the cytoskeletal architecture of the cell (13). Searching for GAP-associated proteins is therefore of particular interest as it may lead to the identification of novel cellular components which may play an important role in Ras regulation and also, in furthering our understanding of the mechanisms by which oncogenic tyrosine kinases may effect transformation. Here we report the cloning and characterization of a novel GAP-binding protein, mTid-1, a DnaJ chaperone protein that represents the murine homolog of the *Drosophila* tumor suppressor *l(2)tid* gene (17) and the recently identified human Tid (*TIDI*) gene (18).

The ubiquitously expressed DnaJ family of proteins serve as regulatory factors to the evolutionary conserved heat shock 70 (Hsp70) superfamily of molecular chaperones (19, 20). This protein family is defined by a highly conserved J-domain, which functions as the binding region for Hsp70 chaperones and orchestrates their interaction with specific substrates (21).
Hsp70 proteins and their associated DnaJ co-chaperone mediate a variety of cellular activities including the folding of newly synthesized polypeptides, the translocation of proteins across membranes, and assembly of multimeric protein complexes (22–25). More recently, genetic and biochemical studies have implicated DnaJ and Hsp70 proteins as important components of intracellular signaling pathways linked to cell survival and growth regulation. In this context, they regulate many facets of the signaling process that have been described for protein modules such as pleckstrin homology SH2 and SH3 domains, namely subcellular localization, regulation of enzymatic activity, and enzyme/substrate recognition (5). For example, genetic studies of v-src toxicity in yeast indicate that the DnaJ protein, Ydj1, is necessary for the correct subcellular targeting and kinase activation of v-src (26, 27). Ydj1 has also been implicated as a positive regulator of cell cycle progression essential for efficient recognition and phosphorylation of cyclin CLN3 by cdc28, events that signal CLN3 degradation (28). Additional biochemical evidence suggests that members of the DnaJ and Hsp70 family interact with and modulate the growth-suppressive properties of several tumor suppressor proteins, including p53, Wilms’ tumor suppressor (WT1), retinoblastoma (Rb) and the double-stranded RNA-activated protein kinase PKR (29–36).

The *Drosophila* l(2)tid gene is the first member of a DnaJ chaperone family to be classified as a tumor suppressor (17). Recessive mutations at the l(2)tid locus cause defects in differentiation and morphogenesis of larval imaginal discs leading to neoplastic growth of these cells into lethal tumors. hTid-1, the human homologue of the l(2)tid-encoded protein Tid65, was recently isolated in a screen for cellular substrates of the human papillomavirus E7 oncoprotein (18). Two splice variants, hTid-1L and hTid-1S, have been identified. Interestingly, although both have been reported to localize to the mitochondria, they seem to display opposing effects on apoptosis. Notably, hTid-1S suppresses cytochrome c release and caspase 3 activation in response to tumor necrosis factor α stimulation, whereas hTid-1L enhances the apoptotic effects of the tumor necrosis factor α receptor (37).

In this study, we demonstrate that mTid-1, a novel member of the Tid family of chaperone proteins, complexes with GAP both in vitro and in vivo in a phosphorylation-independent fashion. Immunofluorescence experiments reveal that Tid-1 proteins are localized to the cytosol, mitochondria, or nucleus, depending on the particular cell type. Moreover, in response to epidermal growth factor stimulation, GAP and Tid-1 colocalize to distinct perinuclear subdomains resembling mitochondrial membranes. Furthermore, we present evidence that Tid-1 can associate with both cytoplasmic and mitochondrial Hsp70 chaperones. These findings suggest a possible novel role for GAP in collaboration with Tid-1 in the integration of mitogenic-signaling pathways at the plasma membrane and control of apoptotic signal transduction at mitochondrial membranes.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—NIH 3T3 mouse fibroblasts, Rat2 fibroblasts, COS-1 monkey kidney, SAOS-2 human osteosarcoma, and MCF-7 breast cancer cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (Life Technologies). SA7a cells (v-src transformed Rat-2 fibroblasts) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies). All cells were maintained in a humidified incubator at 37 °C and 5% CO₂.

**Antibodies**—Mouse monoclonal antibodies to phosphotyrosine (PY20) and p120 RasGAP (GAP) were purchased from Transduction Laboratories (Lexington, KY); mitochondrial Hsp70 (Grp 75, M3-028) antibodies were from Affinity Bioreagents (Golden, CO). Rat monoclonal Hsc70 (SPA-815) and rabbit polyclonal Hsp70 (SPA-812) antibodies were obtained from Stressgen Corp. (Victoria, BC). Mouse monoclonal 2G2 antibody (M00129) to an as yet uncharacterized integral membrane protein of the inner mitochondrial membrane was obtained from Bioo Sciences (Toronto, ON). Rabbit antisera raised against a bacterial fusion protein TrpE-GAP (GAP amino acid residues 171–448) was kindly provided by T. Pawson. Polyclonal rabbit antibodies to murine Tid-1 were generated using a glutathione S-transferase fusion protein expressing the C-terminal 91 amino acid residues (389–480) of mTid-1. Affinity purification of mouse Tid-1 antibodies for immunofluorescence was carried out as described (38).

**Plasmids and Transfections**—The cDNAs encoding full-length mTid-1L, (amino acid residues 1–480), mTid-1I (residues 1–453), and mTid-1S (residues 1–429) were subcloned into pcDNA3 and pcDNA3-MYC expression vectors (Invitrogen). Transient transfections were performed by calcium phosphate-DNA precipitation methods.

**In Vitro Binding Assay**—M3.2 cDNA encoding mouse Tid-1, sequences (amino acid residues 9–453) was in vitro transcribed and translated using a T’N’T kit (Promega) with T7 polymerase according to the manufacturer’s recommendations. Radiolabeled antibodies (8 μl) derived from reactions either containing or lacking M3.2 cDNA as template were incubated for 1 h at 4 °C with anti-mTid-1 antibodies. Labeled M3.2 proteins were also mixed with 1 ml of SA7a cell lysate, prepared in PLC buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprozin), and incubated for 1 h at 4 °C with polyclonal antibodies to GAP or with normal rabbit serum as a control. Immune complexes were washed three times with PLC buffer and resolved by SDS-polyacrylamide gel electrophoresis. Radiolabeled proteins were visualized by autoradiography.

**Immunoprecipitations and Western Blot Analysis**—Cells were washed twice with PBS and lysed in ice-cold PLC buffer. Before lysis and immunoprecipitation with Hsp70 antibody, NIH 3T3 cells were heat-shocked for 1 h in a water bath heated to 43 °C and returned to 37 °C for 6 h to allow for Hsp70 accumulation. For immunoprecipitation experiments with Hsc70, cells were lysed in buffer that either contained or lacked 10 mM ATP. All cell lysates were normalized for protein content before immunoprecipitation with the corresponding antibody. Typically, 1 mg of protein was used in immunoprecipitations, and 50 μg of protein was used for whole cell lysates. Proteins were separated by electrophoresis on an 8% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Roche Molecular Biochemicals). After incubation with the respective primary antibody and subsequently with the corresponding horseradish peroxidase-conjugated secondary antibody, proteins were detected using ECL reagent (Amersham Pharmacia Biotech).

**Northern Blot Analysis**—Total RNA was extracted from FVB mouse...
tissues using TRIzol reagent (Life Technologies), separated on a 1%
formaldehyde-agarose gel, and blotted onto a
\( \text{-probe} \) membrane (Bio-
Rad). A 733-base pair fragment corresponding to nucleotides 690–1482
of mouse Tid-1I cDNA labeled with \([\text{32P}]\text{dCTP} \) (PerkinElmer Life Sci-
ences) was used to probe the blot. Mouse glyceraldehyde-3-phosphate
dehydrogenase served as a quantitative control. Membranes were
stripped and reprobed for mouse glyceraldehyde-3-phosphate dehydro-
genase as control. Hybridizations were carried out according to the
method of Church and Gilbert (39).

**Immunofluorescence**—NIH 3T3, SAOS-2, and COS-1 cells were
seeded into six-well plates containing glass coverslips and allowed to
adhere overnight. COS-1 cells transiently transfected with mTid-1 ex-
pression plasmids were plated 24 h after transfection. For mitochon-
drial staining with rhodamine 123 (Molecular Probes Inc.), cells were
incubated 10 min with a 1:4000 dilution of a 2.5 mM stock in PBS.
Culture media was then added, and the cells were incubated for an
additional 30 min at 37 °C. Staining of mitochondria with Mito-
Tracker® mitochondrion-selective dye (Molecular Probes Inc.) was done
as per the manufacturer’s instructions using a working concentration of
50 nM. Slides were washed with PBS then subjected to fixation in 4%
paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4 °C.
After three washes with PBS, cells were permeabilized with 0.5% Tri-
ton X-100 in PBS for 5 min, rinsed, and blocked in 5% normal goat
serum (Vector Laboratories) in PBS for 30 min at room temperature.
Slides were then incubated for 1 h at room temperature in 5% goat
serum containing affinity-purified mTid-1 antibody at 15 \( \mu \text{g/mL} \). For
double-labeling, a mixture of mTid-1 antibody and mitochondrial 2G2
antibody was used. After three washes with PBS, cells were incubated
for 1 h at room temperature with a mixture of Alexa™488 goat anti-
rabbit IgG and Alexa™546 goat anti-mouse IgG (Molecular Probes Inc.),
each diluted 1:200 in PBS. For nuclei staining, cells were incu-
bated for 10 min with a 1:100 dilution of 10 \( \mu \text{g/mL} \) Hoechst stain. Cells
were viewed and photographed using a Leitz Diaplan fluorescent
microscope.

**RESULTS AND DISCUSSION**

**Identification of mTid-1 as a GAP-binding Protein**—In an
attempt to identify binding partners of GAP, we tested pro-
ducts of cDNA clones that had been isolated from a mouse mammary cDNA library in a separate screen for novel mouse tissues. A single transcript of \( \sim 2.6 \) kilobases was highly expressed in each tissue examined. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was
used as a loading control.

**FIG. 2.** Sequence alignments and schematic representation of three isoforms of mouse Tid-1. A, amino acid sequence of mouse Tid-1
(mTid-1; GenBank™ accession number AY009320) in comparison with Drosophila Tid56 and human Tid-1 (hTid-1). Shaded areas mark identical
amino acid residues. Boxes highlight the mitochondrial signal sequence and the CXCGXXG repeats. B, structural organization of the three
isoforms of mouse Tid-1 isolated from a mouse thymus cDNA library. Boxes highlight the structural domains of Tid-1. The positions of amino acid residues flanking the boundaries of each region are indicated. C, Northern blot analysis of mTid-1 mRNA expression in various mouse tissues. A single transcript of \( \sim 2.6 \) kilobases was highly expressed in each tissue examined. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was
used as a loading control.
targets of oncogenic v-src tyrosine kinase (data not shown) for their ability to bind to GAP in vitro. The product of one clone, M3.2, spanned an open reading frame of 445 amino acids and exhibited a high degree of sequence identity with the protein product of the Drosophila tumor-suppressor imaginal disc (2)tid gene, Tid56 (17), and the recently identified human TID1 gene, hTid-1 (18). We refer to this murine homologue of hTid-1 as mTid-1. The ability of mTid-1 to interact with GAP was tested in vitro by determining whether proteins of in vitro transcribed and translated M3.2 cDNA could interact with GAP present in lysates from v-src-transformed Rat2 fibroblasts (S7a). As shown in Fig. 1, a single radiolabeled translation product of ~48 kDa was immunoprecipitated by mTid-1-specific antibodies raised against the C-terminal residues of M3.2 expressed as a glutathione S-transferase fusion protein from rabbit reticulocyte lysates containing M3.2 cDNA (lane M3.2) but not in the control lysate lacking a DNA template (lane C). The 48-kDa protein was also observed in GAP immune complexes after incubation of [35S]methionine-labeled M3.2 translation products with S7a lysates, suggesting that mTid-1 is capable of associating with GAP proteins.

Deduced Amino Acid Sequence of Mouse Tid—Full-length mouse Tid-1 cDNA was obtained by further screening of a mouse thymus cDNA library using M3.2 as a probe. As shown schematically in Fig. 2A, both mouse and human Tid-1 cDNAs encode proteins of 480 amino acids, with a predicted molecular mass of 53 kDa. Overall, mouse and human Tid-1 proteins are 87.5% identical, with the lowest degree of similarity residing in the first 88 amino acids. Analysis of the deduced protein sequence of mTid-1 revealed typical modular features common to DnaJ chaperone proteins, including an N-terminal J-domain, displaying ~95 and 69% identity with that of hTid-1 and Drosophila Tid56, respectively; a glycine/phenylalanine-rich hinge region, separating the J-domain from a zinc finger-like region. This contains four repeats of the sequence CXXCXXG (where X denotes any amino acid) and is thought to stabilize the interaction of the target substrates with the Hsp70 chaperone machinery (41); and a less conserved C-terminal domain, exhibiting ~92 and 39% identity with hTid-1 and Drosophila Tid56, respectively, thought to be involved in substrate binding. Flanking the N terminus of the J-domain is a mitochondrial cleavage motif (LRP-GV) (42) common to hTid-1 and other polypeptides targeted to the mitochondrial matrix. This would suggest murine Tid-1 proteins, like their Drosophila and human counterparts, may also be localized to the mitochondria.

Mouse Tid-1 Gene Encodes Three Alternatively Spliced Isoforms—Three alternatively spliced variants of mouse Tid-1 were isolated from a mouse thymus cDNA library (Fig. 2B). The long form, mTid-1L, corresponds to the full-length 480-amino acid protein. In the intermediate form mTid-1, the C-terminal amino acids 447–480 of mTid-1L, corresponding precisely to exon 11 of the human TID1 gene, are spliced and replaced with six amino acids, KRSTGN, located within a downstream exon. Notably, the partial M3.2 cDNA clone spans amino acid residues 9–453 of mTid-1L. Both the long and intermediate forms of mTid-1 agree with the previously reported human hTid-1L and hTid-1S alternative splice variants (37), which we will refer to herein as hTid-1L and hTid-1S, respectively. The third and shortest variant, mTid-1S, contains an in-frame deletion of 50 amino acids (codons 211–260). This deletion, which corresponds precisely to exon 5 of the human TID1 gene, results in a loss of two of the four CXXCXXG motifs. Although DnaJ proteins containing only two cysteine-rich repeats have yet to be identified, DnaJ homologs lacking the zinc finger motif altogether have already been characterized and shown to exhibit some chaperone activity (17, 21, 43). The expression pattern of mTid-1 mRNA in several mouse tissues was determined by Northern blot analysis using a mTid-1 cDNA fragment (nucleotides 690–1482) as a probe. As depicted in Fig. 2C, a single transcript of ~2.6 kilobases is highly expressed in each tissue examined. This contrasts with the variable expression observed of hTid-1 mRNA in human tissues, where strong signals are detected primarily in heart, liver, and skeletal muscle (18). A prominent band of ~1.9 kilobases is also evident in mouse tests. The identity of this mRNA species is not known. Since transcripts encoding mTid-1L, mTid-1S, and mTid-1S differ by less than 150 nucleotides, it is not possible to resolve individual mRNA species by Northern blot analysis.

To detect endogenous mTid-1 proteins, cell lysates from Rat2 (R2) fibroblasts were immunoprecipitated and immunoblotted with polyclonal antibodies raised to mTid-1. Two major forms of mTid-1, migrating as a doublet with apparent molecular sizes of 43 and 40 kDa, were identified (Fig. 3A). Interestingly, in several tumor cell lines screened by Western blot analysis of whole cell lysates, an additional band was observed with an approximate molecular mass of 46–48 kDa (Fig. 3B and data not shown). Ectopic expression of mTid-1L and mTid-1S in COS-1 cells gave rise to prominent bands that comigrate with the 43- and 40-kDa endogenous forms of Tid-1, respectively (Fig. 3C). Although transfection of the recombinant mTid-1S cDNA construct generated high levels of mTid-1 proteins of the

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*Fig. 3. Two major isoforms of mTid-1 are recognized in cell lysates. A, cell lysates from Rat2 (R2) were immunoprecipitated (IP) with either pre-immune serum or anti-mouse Tid-1 antibodies raised against the C terminus of mTid-1L, separated by SDS-polyacrylamide gel electrophoresis, and immunoblotted with anti-mTid-1 serum. The arrows indicate the 43- and 40-kDa isoforms of murine Tid-1 recognized in cells. B, additional bands of 46 and 48 kDa were detected in osteosarcoma (SaOS-2) and MCF7 breast tumor cell lines, respectively, by Western blot analysis. C, cell lysates prepared from COS-1 cells transfected with long (mTid-1L), intermediate (mTid-1S), and short (mTid-1S) isoforms of mouse Tid-1 cDNA were also subjected to Western blot analysis with anti-mTid-1 antibodies. The apparent molecular size of the resulting polypeptides representing precursor and processed forms of mTid-1 are indicated in the margin.*

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levels were analyzed by probing aliquots with anti-GAP and anti-mTid-1 antibodies (right panel). mTid-1 and GAP levels were determined by Western blot analysis of R2 and S7a lysates with antibodies to mTid-1 and GAP, respectively (left panel). Tyrosine phosphorylation levels of mTid-1 and GAP were determined by Western blot analysis of R2 and S7a lysates with antibodies to phosphotyrosine (pTyr; middle panel). B, anti-GAP and anti-mTid-1 immunoprecipitates (IP) from S7a cell lysates were immunoblotted with anti-phosphotyrosine antibodies (left panel). The corresponding anti-GAP and anti-mTid-1 immunoblots of the same membrane are shown in the right panel. C, COS-1 cells ectopically expressing mTid-1L, mTid-1I, and mTid-1S were subjected to immunoprecipitation (IP) and immunoblot analysis with antibodies to GAP and mTid-1 respectively. Aliquots (50 μg) of whole cell lysates (WCL) were probed with anti-mTid-1 antibodies to confirm comparable expression of mTid-1L, mTid-1I, and mTid-1S (bottom panel).

predicted 38-kDa size, the endogenous short mTid-1S form was not readily observed in any of the cell lysates examined. However, reverse transcription-polymerase chain reaction analysis has revealed that mTid-1S transcripts are selectively expressed in mouse thymus, kidney, and liver (data not shown). Transient expression of the long, intermediate, and short isoforms of mTid-1 also gives rise to additional higher molecular mass polypeptides of apparent molecular sizes of 50, 48, and 46 kDa, respectively (Fig. 3C). As noted above, the mTid-1 protein is characterized by a mitochondrial cleavage signal situated between residues 63 and 67. Because most mitochondrial proteins are nuclear-encoded, they are synthesized as precursors in the cytosol and processed to a mature form upon import into the mitochondria (42, 44). Therefore, the higher molecular mass proteins in the 46–50-kDa range observed in tumor and transfected cells likely represent cytoplasmic precursors of the mature mitochondrial forms. This suggestion is supported by the identification of both cytoplasmic and mitochondrially localized Drosophila (D. melanogaster) Tid50 (36) and Tid56 (37) encoded proteins, believed to represent the Tid56 precursor and mature Tid50 forms, respectively, as revealed by immunoelectron microscopy of Drosophila Schneider 2 cells (45). The significance of the observed overexpression of endogenous precursor protein in tumor cell lines is currently being investigated.

**GAP Associates with mTid-1 in Vivo**—To assess possible in vivo interactions between mTid-1 and GAP, lysates from Rat2 (R2) and v-src-transformed Rat2 fibroblasts (S7a) cells were immunoprecipitated with antibodies directed against phosphotyrosine (pTyr) and immunoblotted with anti-GAP and anti-mTid-1 serum. In both cell lines examined, the endogenous 40-kDa mTid-1I and 43-kDa mTid-1S forms of mTid-1 were found to coprecipitate with GAP (Fig. 4A), suggesting that the interaction between GAP and mTid-1 occurs in a kinase-independent manner. However, analysis of mTid-1 immune complexes with anti-GAP antibodies failed to demonstrate detectable binding between mTid-1 and GAP (Fig. 4B, top panels). One possible explanation is that antibodies to mTid-1 were raised to the C-terminal substrate binding domain and, thus, may preclude binding of GAP to mTid-1.

To determine if mTid-1 is an intracellular target of activated tyrosine kinases, cell lysates prepared from R2 and S7a cells were probed with anti-phosphotyrosine antibodies on Western blots. As shown in Fig. 4A, measurable tyrosine phosphorylation of mTid-1I and mTid-1S proteins was observed in S7a but not R2 cells, suggesting that mTid-1S may serve as a direct or indirect substrate of v-src tyrosine kinase. Intriguingly, immunoblot analysis of GAP immunoprecipitates with anti-phosphotyrosine antibodies revealed that GAP associates predominantly with the unphosphorylated forms of mTid-1 (Fig. 4B, bottom panels). Phosphorylation of mTid-1 may impede its binding to GAP or, alternatively, may function as a trigger for dissociating the mTid-1-GAP complex, analogous to that described for the Hsp90-v-src heterocomplex (46).

The binding of each of the mTid-1 isoforms to GAP was further examined using transient expression assays in COS-1 cells. Lysates from cells transfected with mTid-1L, mTid-1I, and mTid-1S were immunoprecipitated with anti-GAP serum following by immunoblotting with anti-mTid-1 antibodies. GAP was found to associate with both full-length precursor and mature processed forms of all three mTid-1 splice variants (Fig. 4C). Moreover, in cells ectopically expressing mTid-1S, a significant increase in the interaction of GAP with mTid-1S was observed compared with GAP-mTid-1L or GAP-mTid-1I complexes, suggesting preferential binding of GAP to the intermediate isoform of mTid-1S. Recently, it was reported that hTid-1L and hTid-1S have opposing effects on apoptosis, with hTid-1L enhancing and hTid-1S suppressing apoptosis induced by tumor necrosis factor α (37). Interestingly, the relative level of endogenous mTid-1S is elevated in v-src-transformed S7a cells (Figs. 3B, 4A), consistent with the idea that v-src might induce expression of the anti-apoptotic form of mTid-1S. Although the biochemical function of the mTid-1-GAP complex is not known, enhanced binding of mTid-1S to GAP may conceivably exert inhibitory effects on GAP activity. In this context, it is note-
worthy that the persistent association of a Ydj1 mutant with v-src severely compromises the in vivo activity of the kinase without affecting the levels of v-src in the cell (26). Alternatively, increased binding of mTid-1 with GAP may serve to sequester GAP away from Ras. Either of these possibilities could contribute to aberrant cell growth through dysregulation of the Ras-signaling pathway.

mTid-1 Interacts with Multiple Members of the Hsp70 Family of Molecular Chaperones—The highly conserved J-domain of mouse Tid-1 suggests the protein may function as a cofactor of the Hsp70 chaperone machinery. It has recently been demonstrated that hTid-1, and hTid-1 interact specifically with mitochondrial Hsp70 (Grp75) (37). Given that our polyclonal antibodies are immunoreactive to both cytoplasmic and mitochondrial mature forms of mTid-1, whereas those used by Syken et al. (Ref. 37 and data not shown) only recognize the mitochondrial-localized forms of hTid-1, we sought to establish whether mTid-1-encoded proteins also bind nonmitochondrial members of the Hsp70 family. To this end, coimmunoprecipitation assays were performed using antibodies to mTid-1 and antibodies specific to three distinct members of the Hsp70 family, notably mitochondrial Grp75, cytoplasmic heat-shock-inducible Hsp70, and constitutively expressed Hsc70. As reported for hTid-1, endogenous 43-kDa mTid-1L and 40-kDa mTid-1I proteins can form complexes with mitochondrial Grp75 (Fig. 5A). To study the interaction of mTid-1 with stress-inducible Hsp70, NIH 3T3 cells were heat-shocked for 1 h at 43 °C before lysis and immunoprecipitation analysis with anti-mTid-1 or anti-Hsp70 antibodies. A significant induction of Hsp70 protein levels was observed in heat-shocked cell lysates, whereas no concomitant increase in the expression of either endogenous mTid-1L or mTid-1I proteins was observed. As shown in Fig. 5B, cytosolic stress-associated Hsp70 was recovered in association with mTid-1L and mTid-1I upon heat treatment but not in control untreated cells. Anti-Hsp70 antibodies were not as efficient as anti-Grp75 antibodies in coprecipitating mTid-1 proteins. Hsc/Hsp70 protein complex assembly and dissociation is ATP-dependent. Previous studies have shown that the binding of ATP to the ATPase domain of Hsc70 induces conformational changes that modulate the binding of cochaperone proteins (47–49). We therefore examined the ATP dependence of Tid-1 interactions with Hsc70. As shown in Fig. 5C, the addition of 10 mM ATP to cell lysates before immunoprecipitation with anti-Hsc70 antibodies, was found to enhance the association of Tid-1L and Tid-1I with the constitutive cytosolic Hsc70. Taken together, these findings implicate mTid-1 as a regulatory cofactor to members of Hsp70 chaperone family. Furthermore, our observation that mTid-1 interacts with Hsc70 and Hsp70, which localize to nonmitochondrial, cytosolic, and nuclear compartments of the cell, supports the idea that mTid-1 does not function exclusively in mitochondria and that its biological activities in mammalian cells may be dictated by subcellular location and its Hsp70 partner. In this context, it is noteworthy that both hTid-1 and Hsp/Hsc70 proteins have been implicated in cell death suppression (37, 50, 51), raising the possibility that Tid-1 proteins may modulate the chaperone activity Hsp70 family members during the conformational regulation of proteins involved in apoptotic signal transduction. In any case, mTid-1 may be included among a number of mitochondrial-targeted proteins that have been identified to function at specific extramitochondrial locations (53).

We next reasoned that if Tid-1 is functioning as a Hsp/Hsc70 cofactor, then conceivably some of the identified cellular partners of Tid-1 may serve as substrates of Hsp/Hsc70 chaperones. To examine if GAP is recruited to Hsp/Hsc70 chaperone complexes through its association with mTid-1, we performed coimmunoprecipitation assays followed by immunoblot analysis with anti-Hsp/Hsc70 and anti-GAP antibodies. However, we were unable to observe an in vivo association of Hsp/Hsc70 proteins with GAP, possibly due to the transient nature of Hsp chaperone/substrate interactions (data not shown). This is consistent with previous reports indicating that Hsp/substrate heterocomplexes are unstable and easily disrupted by standard cell lysing and immunoprecipitation procedures (49, 52).

Immunolocalization of Endogenous and Ecopically Expressed mTid-1—To further explore the subcellular localization of Tid-1 in mammalian cells, the protein was visualized in several cell lines by immunofluorescence microscopy. In mouse NIH 3T3 cells, mTid-1 was detected both in the cytosol and in the nuclear compartment (Fig. 6A, panel i). The fact that Tid-1 proteins do not possess sequences conforming to a nuclear localization signal suggests that mTid-1 could gain entry into the nucleus through its interactions with nuclear-bound proteins. Indeed, hTid-1 has been reported to interact with the nuclear-localized human papilloma virus E7 oncoprotein (18).
Analysis of the cytosolic-immunostaining pattern of mTid-1 at higher magnification (Fig. 6A, panels iv–vi) revealed association of mTid-1 with cytosolic organelles that morphologically resembled mitochondria (Fig. 6A, panel vi). Immunolocalization of Tid-1 in the human tumor cell line SAOS-2 (Fig. 6B, panel ii) or with a monoclonal antibody (2G2) to an integral membrane protein of the inner mitochondrial membrane and rhodamine-conjugated secondary antibody (C, panel ii). Subcellular localization of the precursor form of mTid-1L (C, panel iii) or processed mTid-1L isoform (C, panel v) was examined using COS-1 transfectants expressing MYC epitope-tagged or untagged mTid-1L, respectively. Transfected cells are indicated by arrowheads. Nuclei were identified by Hoechst staining.

mTid-1 serum confirmed a predominance of the protein in its unprocessed, full-length form (data not shown). Parallel immunolocalization assays of cells expressing a nonepitope-tagged form of mTid-1L revealed a combination of diffuse cytosolic staining and punctate foci, likely representative of the unprocessed and mature forms of mTid-1 proteins, respectively (Fig. 6C, panel v). The subcellular distribution of the other two isoforms of mTid-1 transiently transfected in COS-1 cells was indistinguishable from that of mTid-1L-encoded proteins (data not shown). Taken together, these data corroborate and further extend previous reports obtained from subcellular fractionation experiments with endogenous hTid-1 and Drosophila Tid56, demonstrating that Tid proteins are predominantly distributed within mitochondrial fractions (37, 45). Interestingly, immunogold electron microscopy with Drosophila Schneider 2 cells indicated that the Tid56 protein can be found in the cytosol as well as associated with both outer and inner mitochondrial compartments of the cell (45). Our data support the notion that the cellular background in which Tid-1 is expressed can influence whether it resides in the cytosol, mitochondria, or is found also in the nucleus.
mTid-1 and GAP Colocalize in Response to EGF—Based on the evidence that mTid-1 and GAP associate in mammalian cells, we reasoned that a portion of GAP proteins should be targeted to the same membranous location where Tid-1 resides. To test this hypothesis, COS-1 cells were either serum-starved or treated with EGF, and the subcellular location of Tid-1 and GAP was determined. Under serum starvation, Tid-1 and GAP exhibited a punctate distribution throughout the cytosolic compartment that showed some degree of overlap (Fig. 7A, panels i–iii). Analysis of the immunofluorescent staining patterns after incubation with EGF for 5 min (Fig. 7B, panels i and ii) revealed extensive colocalization of GAP and Tid-1 to common perinuclear subcellular domains (yellow stain, Fig. 7B, panel iii). The shift in GAP localization in response to EGF is consistent with a previous study demonstrating GAP translocates from the cytosol to perinuclear foci, suggestive of an association with mitochondria upon EGF stimulation (40).

Conclusions—We have identified the mouse homolog of a member of the DnaJ family, hTid-1, as a novel p120 RasGAP-binding protein. DnaJ proteins interact with Hsp70 chaperones to modulate their chaperone activities in specific cellular compartments and cellular protein processes. The variations in the intracellular localization of mTid-1 proteins and the apparent association with both cytosolic and mitochondrial members of the Hsp70 family suggests that the in vivo function of Tid-1 likely depends on cellular context. The localization of mTid-1 to the mitochondria and its association with mitochondrial Hsp70 suggests mTid-1 functions as a regulatory factor to mediate the import and folding of proteins into the mitochondrial matrix. Alternatively, given that both hTid-1 and Hsp70 proteins have been implicated as modulators of apoptosis, we postulate that Tid-1 may cooperate with Hsp/Hsc70 chaperones to promote changes in enzymatic activities, oligomerization states, binding affinities, or intracellular targeting required for the activation of signaling molecules involved in apoptotic signaling at the mitochondrial surface.

The I29/tid gene was originally classified as a tumor suppressor in Drosophila in which recessive mutations lead to malignant transformation of the imaginal discs of the larva (54). Although TID1 has not previously been recognized as a tumor suppressor in humans, it is tempting to speculate on its tumor suppressor function in a mammalian setting as well. Importantly, our data point to a potential role for mTid-1 in GAP-mediated regulation of cell growth. In this capacity, mTid-1-Hsp/Hsc70 heterocomplexes may govern the conformational maturation and/or activity of GAP required for its role as a negative regulator of Ras or as a regulator of cytoskeletal organization (4). mTid-1 may also assist in the assembly of complexes consisting of GAP and other signaling proteins such as p62bik and p190 involved in GAP-directed activities. Alternatively, the association of mTid-1 with GAP may function to sequester GAP from the cytosol to the mitochondria, thereby modulating its interaction with and GTPase-promoting activity towards Ras in response to growth factor receptor activation. At any rate, one may envision that in the absence of functional Tid proteins, GAP may escape the regulation imposed by Hsp70 association and consequently affect the ability of GAP to effectively down-regulate Ras, which may contribute to a hyperproliferative phenotype. Significantly, it has been shown that loss of expression of another Ras GTPase-activating protein, neurofibromatosis-1, in neurofibroma tumors correlates with increased levels of activated Ras (55). Additional investigations of the biochemical function of Tid-1 and GAP complexes may improve our understanding of how Tid-1 DnaJ proteins may exert their effects on cell survival and cell growth.

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A Mouse Homologue of the Drosophila Tumor Suppressor l(2)tid Gene Defines a Novel Ras GTPase-activating Protein (RasGAP)-binding Protein
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