Human T cell leukemia virus type 1 Tax associates with a molecular chaperone complex containing hTid-1 and Hsp70

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Tax, an oncogenic viral protein encoded by human T cell leukemia virus type 1 (HTLV-1), induces cellular transformation of T lymphocytes by modulating a variety of cellular gene expressions [1]. Identifying cellular partners that interact with Tax constitutes the first step toward elucidating the molecular basis of Tax-induced transformation. Here, we report a novel Tax-interacting protein, hTid-1. hTid-1, a human homolog of the *Drosophila* tumor suppressor protein Tid56, was initially characterized based on its interaction with the HPV-16 E7 oncoprotein [2]. hTid-1 and Tid56 are members of the DnaJ family [2, 3], which contains a highly conserved signature J domain that regulates the activities of heat shock protein 70 (Hsp70) by serving as cochaperone [4–6]. In this context, the molecular chaperone complex is involved in cellular signaling pathways linked to apoptosis, protein folding, and membrane translocation and in modulation of the activities of tumor suppressor proteins, including retinoblastoma, p53, and WT1 [7–12]. We find that expression of hTid-1 inhibits the transformation phenotype of two human lung adenocarcinoma cell lines. We show that Tax interacts with hTid-1 via a central cysteine-rich domain of hTid-1 while a signature J domain of hTid-1 mediates its binding to Hsp70 in HEK cells. Importantly, Tax associates with the molecular chaperone complex containing both hTid-1 and Hsp70 and alters the cellular localization of hTid-1 and Hsp70. In the absence of Tax, expression of the hTid-1/Hsp70 molecular complex is targeted to perinuclear mitochondrial clusters. In the presence of Tax, hTid-1 and its associated Hsp70 are sequestered within a cytoplasmic "hot spot" structure, a subcellular distribution that is characteristic of Tax in HEK cells.

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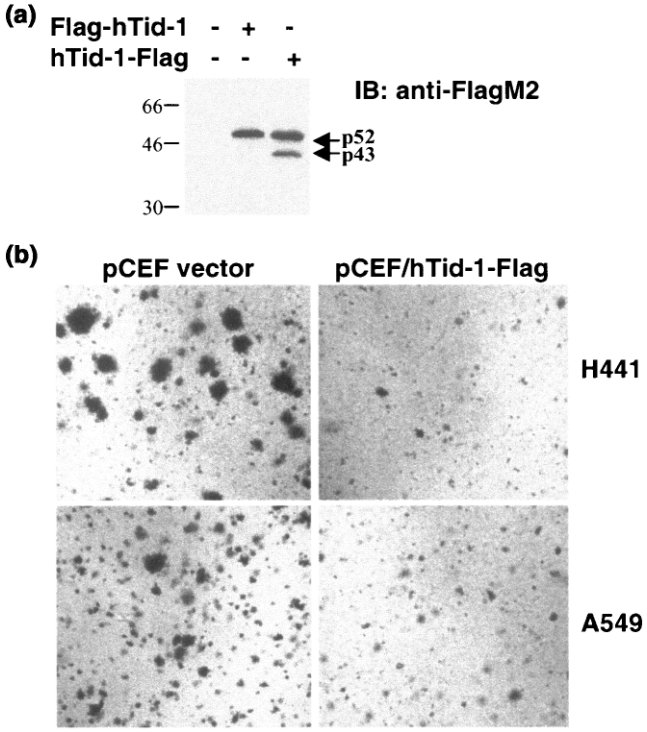
Results and discussion

In order to gain a better understanding of the molecular mechanism of Tax-mediated cellular transformation, we utilized the yeast two-hybrid system to identify potential cellular targets for the HTLV-1 Tax protein. A cDNA sequence was recovered by the use of the transactivation-minus-Tax mutant protein M318 [13] as bait. This sequence appeared to be hTid-1, which encodes a human homolog of the *Drosophila* tumor suppressor Tid56 [2]. Transient expression of C-terminally tagged hTid-1-Flag in HEK cells revealed two forms with apparent molecular masses of 52 Kd (p52) and 43 Kd (p43) (Figure 1a). In contrast, the N-terminally tagged Flag-hTid-1 was visualized as a single p52 form, suggesting the presence of a protease cleavage site located at the N-termini [12]. Thus, it appears that, as in Tid56, the full-length p52 of hTid-1 represents an hTid-1 precursor and that p43 represents a processed form. To examine whether hTid-1 displays tumor suppressive activity, we transfected hTid-1-Flag into two human cancer cell lines (H441, A549) and determined the transforming activity of these two cancer cell lines by their ability to grow in soft agar. We found that in H441 and A549 cells transfected with pCEF vector, colony formation in soft agar could be visualized at day 14 (Figure 1b). However, in cells transfected with pCEF/hTid-1-Flag, colony formation was significantly suppressed (Figure 1b). These findings suggest that hTid-1, similar to *Drosophila* Tid56, has an inhibitory effect on the transforming phenotype of human cancer cells.

The interaction of Tax and hTid-1 was evaluated further in mammalian HEK cells. The coprecipitation of hTid-1 and Hsp70 was also assessed since DnaJ proteins were defined based on their interaction with heat shock proteins through a conserved J domain [5]. Figure 2a schematically depicts the various functional regions of hTid-1. In addition to a signature J domain (amino acids 89–169), hTid-1 contains an N-terminal mitochondrial processing

Figure 1

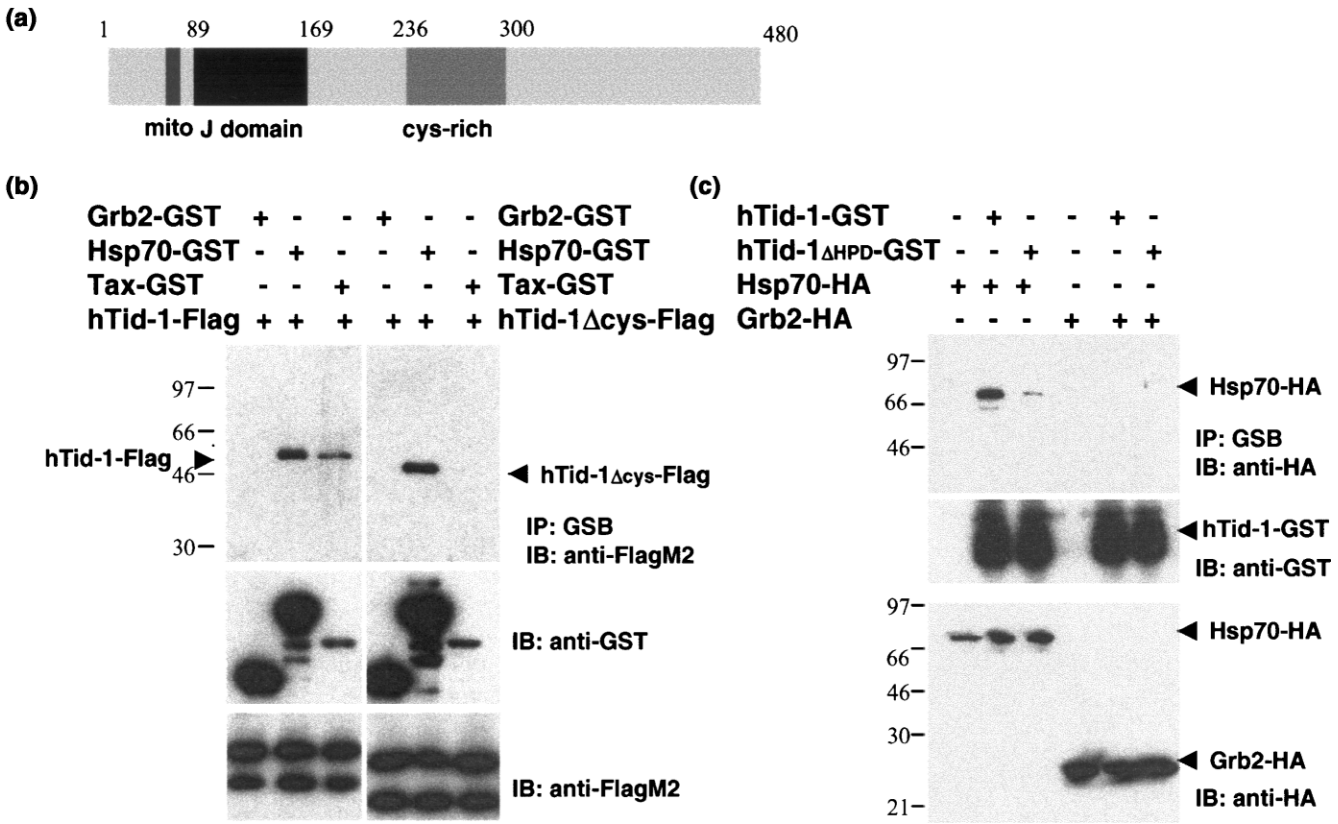


Expression- and transformation-suppressive activity of hTid-1. **(a)** Expression of N- and C-terminally tagged hTid-1 in transfected HEK cells was analyzed with anti-FlagM2 immunoblotting. **(b)** hTid-1 suppresses the in vitro transformation of two human cancer cell lines H441 and A549. Cells were transfected with pCEF/hTid-1-Flag or with the pCEF vector (0.4 μg each) alone. Colony growth in soft agar was assayed as described in the supplementary material. The pictures are representatives of two independent experiments.

peptide sequence 63LRPGV67 and a central cysteine-rich or zinc finger-like region containing four CXXCXGXG repeats (amino acids 236–300). As shown in the top left panel of Figure 2b, hTid-1-Flag, in its p52 form, coprecipitated with Tax-GST and Hsp70-GST, but not with Grb2-GST. Significantly, a mutant hTid-1 (hTid-1ΔCys) with a deletion in the central Cys-rich region (Δ236–292 amino acids) lost its ability to bind to Tax, but it was still able to associate with Hsp70 (Figure 2b, top right panel). As expected, the association of hTid-1 and Hsp70 requires the J domain of hTid-1 (Figure 2c). An hTid-1 mutant with a deletion of the conserved 121HPD123 motif within the J domain was impaired in its ability to coprecipitate Hsp70. Collectively, our data suggest that Tax and Hsp70 bind to different regions of hTid-1, with Tax binding to the Cys-rich domain and Hsp70 interacting with the J domain. The finding that Tax and Hsp70 interact with different domains of hTid-1 suggests that Tax may associate with a molecular chaperone protein complex containing both hTid-1 and Hsp70. As shown in Figure 3, both hTid-1 and Hsp70 could be detected in the Tax-GST precipitates (lane 3). The presence of Hsp70-HA in Tax-GST precipitates of cells cotransfected with hTid-1ΔCys-Flag appeared to be mediated through endogenous hTid-1 since hTid-1ΔCys-Flag was absent in the complex (lane 7). These findings indicate that Tax/hTid-1/Hsp70 form a supercomplex.

hTid-1 has been reported to localize predominantly within the mitochondrial matrix, whereas Tax is localized in both the cytoplasm and the nucleus [12, 14–18]. However, immunofluorescent staining showed that mouse Tid-1 (mTid-1) is present in the mitochondria, cytoplasm, and nucleus [19]. We examined subcellular localizations of fluorescent fusion proteins of hTid-1, Tax, or Hsp70 expressed in HEK cells. As shown in Figure 4a, hTid-1-EGFP displayed a faint punctate perinuclear presence but was also distributed diffusely in the cytoplasm. Tax-RED was found predominantly in the cytoplasm within a unique "hot spot" in HEK cells, whereas Hsp70-RED was diffusely distributed in the cytoplasm with a punctate pattern. The control protein dsRED-mito was visualized distinctly in punctate clusters of the perinuclear mitochondria. The effect of Tax and Hsp70 expressions on the subcellular localization of hTid-1 was further examined with two-color fluorescence microscopy (Figure 4b). In Tax- and hTid-1-coexpressing cells, hTid-1-EGFP (top left) was localized in a unique cytoplasmic "hot spot" distribution pattern that is characteristic of Tax-RED (top right) but is distinct from the relatively diffuse cytoplasmic distribution in cells cotransfected with RED (bottom left). In cells cotransfected with hTid-1-EGFP and Hsp70-RED, both the green (middle left) and red (middle right) fluorescent colors were concentrated in punctate perinuclear clusters, a pattern reminiscent of the subcellular distribution pattern of dsRED-mito (Figure 4a, lower right). These results suggest that Tax could sequester hTid-1 to high concentrations within a specific cytoplasmic "hot spot" structure that is characteristic of Tax localization in HEK cells. Furthermore, the interaction of hTid-1 and Hsp70 could lead to the redistribution of both proteins to form perinuclear mitochondrial clusters. To exclude possible fluorescence interference of EGFP and RED, hTid-1-EGFP was cotransfected with nonfluorescent proteins Tax-HA and Hsp70-HA. The use of HA-tagged Tax and Hsp70 proteins further controled for any spurious effects that fusion to heterologous proteins such as RED might have on their subcellular localizations. As shown in Figure 4c, a typical "hot spot" pattern of hTid-1-EGFP was seen when cells were cotransfected with Tax-HA (upper left), whereas a typical punctate perinuclear mitochondria distribution of hTid-1-EGFP was visualized in cells cotransfected with Hsp70-HA (upper right). As shown by the additional controls in the lower panels of Figure 4c, hTid-1ΔCys-EGFP lost "hot spot" localization when cotransfected with Tax-HA (left), but it still dis-

Figure 2

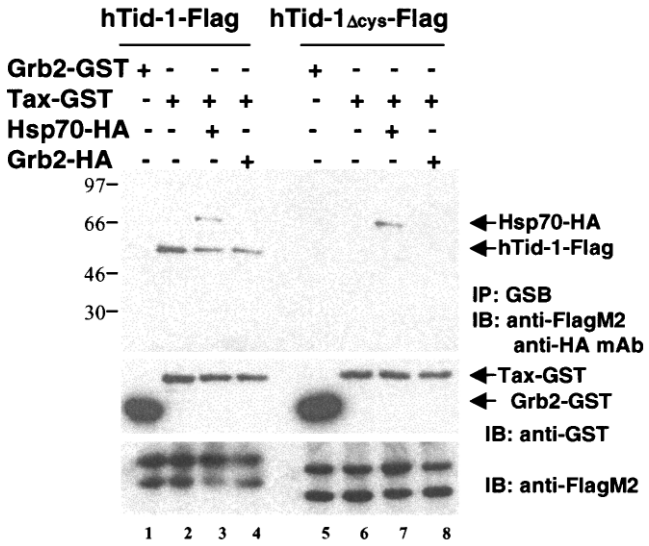


Interaction of hTid-1 with Tax and Hsp70 in vivo. **(a)** Schematic representation of hTid-1. A mitochondrial processing peptide sequence 63LRPGV67 (mito), a signature DnaJ domain (amino acids 89–169), and a central cysteine-rich region (amino acids 236–300) were indicated. **(b)** A central cysteine region of hTid-1 is essential for association with Tax. hTid-1-Flag or hTid-1ΔCys-Flag with an internal deletion of the cysteine-rich domain (amino acids 236–292) (1 μg each) was cotransfected with Grb2-GST, Hsp70-GST, or Tax-GST (1 μg each), and the cellular extracts were precipitated with GSB followed by an anti-Flag blot (top panel). The membrane was stripped and reblotted with anti-GST-HRP (middle panel). The expression of hTid-1 and the mutant in total cell lysates was detected with anti-FlagM2. **(c)** The J domain of hTid-1 mediates its interaction with Hsp70. Hsp70-HA (1 μg) was cotransfected with or without hTid-1-GST or hTid-1ΔHPD-GST (1 μg each) in HEK cells. Grb2-HA was used as a control. Cellular protein extracts were precipitated with GSB followed by an immunoblot with anti-HA (top panel). The membrane was reblotted with anti-GST-HRP (middle panel), and the bottom panel showed total lysates probed with anti-HA.

played the punctate perinuclear distribution when cotransfected with Hsp70-HA (right). These results confirm the findings obtained from two-color fluorescent fusion proteins (Figure 4b) and suggest that hTid-1 can be translocated to different cellular compartments depending on its association with specific cellular partners. Lastly, to assess the effect of Tax on subcellular location of the molecular chaperone complex, we examined the fluorescence distribution of hTid-1-EGFP and Hsp70-RED in the presence and absence of Tax. In agreement with the findings above, coexpression of hTid-1-EGFP and Hsp70-RED resulted in a perinuclear distribution of both proteins (Figure 4d, upper panels). In the presence of Tax, however, a green and red "hot spot" characteristic of that of the subcellular distribution pattern of Tax was formed (lower panels). Taken together, our results indicate that Tax is a determining factor in sequestering the molecular chaperone complex in the cytoplasmic "hot spot."

Members of the DnaJ protein family of molecular chaperones, through their interaction with Hsp70 family members, are known to regulate the activities of tumor suppressors such as WT1 and p53. Similarly, we show here that hTid-1 potently represses the in vitro transforming activity of two human lung adenocarcinoma cell lines. Furthermore, oncogenic viruses such as polyomavirus and SV40 encode large tumor antigens that contain a J domain that is important for inactivating the functions of the Rb family of tumor suppressors [20–22]. HTLV-1 Tax, like the HPV16 E7 oncoprotein, does not contain a J domain. However, it is conceivable that through binding to a J

Figure 3



Tax associates with a molecular chaperone protein complex. Transient transfection in HEK cells was performed with combinations of various constructs (1 μg each) as indicated in the figure. The cellular protein extracts were precipitated with GSB followed by an immunoblot with combined anti-FlagM2 and anti-HA (top panel). The membrane was stripped and reblotted with anti-GST-HRP (middle panel), and total lysates were probed with anti-FlagM2 for the detection of hTid-1-Flag and its mutant (bottom panel).

domain-containing protein such as hTid-1, Tax and E7 could modulate the activity of the molecular chaperone and, as a result, diminish their potential functions as tumor suppressors. Further studies will be required for demonstrating a functional role of the molecular chaperone complex in mediating the transforming ability of Tax.

Supplementary material

Supplementary material showing methodological details as well as the expression of Tax-EGFP and Tax-RED in other cell types is provided with the electronic version of this article at http://images.cellpress.com/supmat/supmatin.htm.

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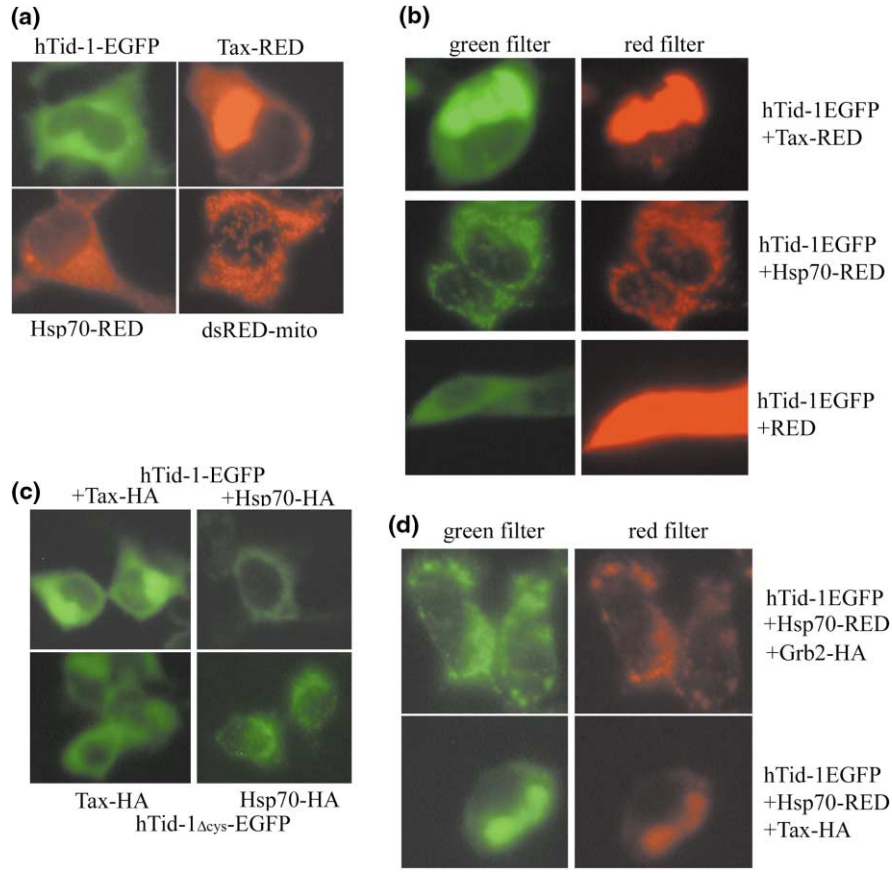
Figure 4



(a) Subcellular localization of hTid-1-EGFP, Tax-RED, and Hsp70-RED fluorescent fusion proteins. Transient transfection of these fluorescent fusion protein constructs (1 μg each) in HEK cells was performed, and the fluorescent signals in live cells were examined with a fluorescence microscope. **(b)** Colocalization of hTid-1 with Tax and Hsp70. hTid-1-EGFP (1 μg) was cotransfected with Tax-RED, Hsp70-RED, or RED (1 μg each) in HEK cells. The green filter detects hTid-1 EGFP, and the red filter detects red proteins, including Tax (top panel), Hsp70 (middle panel), and RED (bottom panel) in the same field for each panel. **(c)** Colocalization of Tax and hTid-1 requires their physical association. hTid-1-EGFP (upper panels) or hTid-1ΔCys-EGFP (lower panels) (1 μg each) was cotransfected with nonfluorescent genes that express Tax-HA and Hsp70-HA (1 μg each). The patterns of the cellular distribution of hTid-1-EGFP and hTid-1ΔCys-EGFP in these cotransfected HEK cells are shown. **(d)** Tax sequesters the molecular chaperone complex in a cytoplasmic "hot spot." hTid-1-EGFP and Hsp70-RED (1 μg each) were transfected together with Grb2-HA as a control (upper panel) or Tax-HA (lower panel) in HEK cells. Typical fluorescent patterns are shown.