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Advances in Brief

Perturbation of TSG101 Protein Affects Cell Cycle Progression[1]

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Abstract

***tsg101* was recently identified as a tumor susceptibility gene by functional inactivation of allelic loci in mouse 3T3 fibroblasts. Although previous studies suggested that homozygous intragenic deletion of *TSG101* is rare in breast cancer cells and specimens, the neoplastic phenotype caused by tsg101 inactivation implicated that tsg101 may play a significant role in cell growth control. Here, we characterize mouse polyclonal and monoclonal antibodies that specifically recognize the TSG101 protein (molecular mass, 46 kDa) in whole-cell lysates by straight Western blot analysis. By indirect immunofluorescence staining, TSG101 was found to be localized in the cytoplasm throughout the entire cell cycle. However, the nuclear staining increases from G_1 to S phase and becomes dominant in late S phase. TSG101 is mainly distributed surrounding the chromosomes during M phase. The expression level of TSG101 is not cell cycle dependent. It is possible that the relocalization of TSG101 from the cytoplasm into the nucleus may be relevant to its function. Microinjection of both polyclonal and monoclonal antibodies specific to TSG101 into cells during G_1 or S phase results in cell cycle arrest. Furthermore, overexpression of TSG101 leads to cell death, suggesting that the appropriate amount of TSG101 is critical for cell cycle progression. Taken together, these results suggest that neoplastic transformation caused by TSG101 deficiency may result from bypassing of the cell cycle checkpoints.**

Introduction

tsg101 is a tumor suppressor gene that was recently discovered using a novel strategy of regulated antisense RNA initiation within a retrovirus-based gene search vector to identify previously unknown autosomal genes whose inactivation is associated with a defined phenotype (1). In this case, functional knockout of *tsg101* in mouse fibroblasts leads to transformation and the ability to form metastatic tumors in nude mice. The cellular transformation and tumorigenesis that result from inactivation of *tsg101* are reversible by deletion of the transactivator gene required for the production of antisense transcripts complementary to *tsg101* mRNA (1). The mechanisms by which interference with TSG101 expression leads to neoplastic transformation remain unclear.

Sequence analysis of both the mouse and human *TSG101* cDNAs indicates that the gene encodes a 43-kDa protein containing a proline-rich domain and a leucine heptad repeat (coiled-coil) domain (1). The coiled-coil domain of TSG101 was found to interact with the cytosolic phosphoprotein stathmin (2). This protein was known as oncoprotein 18 and may have a role in cell growth and differentiation (3, 4). Evidence also suggests that TSG101 may function in both the nucleus and the cytoplasm. In unsynchronized WERI-27 cells, TSG101 was found to be located predominantly in the cytoplasm and may translocate to the nucleus in late S phase or G_2 phase (5). Furthermore, structural analysis of the amino-terminal domain of TSG101 indicates that it mimics a group of ubiquitin-conjugating enzymes, implying that TSG101 may function in ubiquitin-mediated proteolysis and cell cycle progression (6, 7).

Here, we have developed specific mAbs[3] as tools to study TSG101 protein in cells. TSG101 is present in the cytoplasm throughout the cell cycle, whereas nuclear staining for TSG101 increases from G_1 to S phase and appears to be prominent in late S phase. In mitosis, TSG101 is mainly localized to areas surrounding the condensed chromosomes. Consistent with the staining data, overexpressed GFP-tagged TSG101 had both cytoplasmic and nuclear distribution. The expression level of TSG101 is constant throughout the cell cycle. Microinjection of specific anti-TSG101 antibodies into cells during G_1 and S phase arrested cells before M phase. Overexpression of TSG101 causes cell death. These results indicate that TSG101 protein may be crucial for cell cycle progression.

Materials and Methods

Preparation of Polyclonal and Monoclonal TSG101 Antibodies. Mouse monoclonal and polyclonal anti-TSG101 sera were generated using the purified GST-TSG101 fusion protein encoding amino acids 167–374 expressed in bacteria as immunogens, as described previously (5). The polyclonal antiserum from the immunized mice was preabsorbed with GST beads and used directly for immunoprecipitation, immunoblotting, and immunostaining. For microinjection, the polyclonal anti-TSG101 antibodies were further purified using a protein G-Sepharose column. Preimmune serum was obtained from the same mice and used at the same dilution (1:1000). mAbs were prepared according to standard procedures (8) and purified by protein G-Sepharose chromatography before use.

Cell Cycle Synchronization. Human T24 bladder carcinoma cells were synchronized in G_0 by density arrest in DMEM supplemented with 10% FCS and then released at time 0 by replating in the same medium at a density of 2×10^6 cells/10-cm plate. At various time points thereafter (18 h for G_1-S phase, 24 h for S phase, and 33 h for G_2-M phase), cells were harvested. To obtain cells in M phase, nocodazole (0.4 μg/ml) was added to culture medium for 10 h before harvesting the cells. Samples of cells were fixed in ethanol and analyzed using fluorescence-activated cell sorting to determine cell cycle phases as described previously (9).

Immunoprecipitation and Western Blot Analysis. Cells lysed in Lysis 250 buffer were subjected to five freeze/thaw cycles (liquid nitrogen, 37°C) and clarified by centrifugation (14,000 rpm for 2 min at room temperature). The supernatants were then used for immunoprecipitation as described previously (10). Briefly, 1 μl of mouse polyclonal antibody or anti-TSG101 mAb was added to each clarified supernatant. After a 1-h incubation, protein A-Sepharose beads were added and incubated for another hour. The beads were then collected and washed five times with lysis buffer containing 250 mM NaCl and then boiled in SDS loading buffer for immunoblotting analysis as described previously (10).

Immunostaining. The procedure for indirect immunofluorescence staining was adapted as described previously (11). Briefly, cells grown on coverslips in tissue culture dishes were washed in PBS and fixed for 30 min in 4% formaldehyde in PBS with 0.5% Triton X-100. After treating with 0.05% saponin in water for 30 min and extensive washing with PBS, cells were blocked in PBS containing 10% normal goat serum. A 1-h incubation with the

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[3] The abbreviations used are: mAb, monoclonal antibody; GFP, green fluorescence protein; DAPI, 4′,6-diamidino-2-phenylindole; GST, glutathione S-transferase.

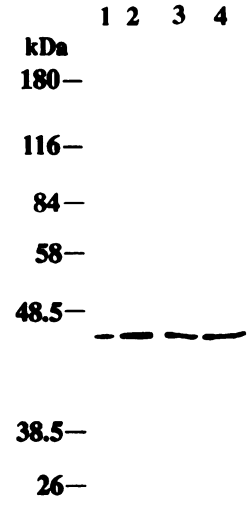

Fig. 1. Characterization of mouse polyclonal antibodies and mAbs. One of the mouse mAbs (4A10) was generated against the same antigen used to make polyclonal anti-TSG101 antibody, and both mouse polyclonal antibodies (*Lanes 1* and *2*) and mAbs (*Lanes 3* and *4*) were used for straight immunoblotting of protein lysates from 2.5×10^5 (*Lanes 1* and *3*) or 5×10^5 T24 cells (*Lanes 2* and *4*).

suitable antibody diluted in 10% goat serum was followed by five washes and then by another 1-h incubation with fluorochrome-conjugated secondary antibody. The antigen was then visualized with goat antimouse antibody conjugated to fluorescein isothiocyanate. After washing extensively in PBS with 0.5% NP40, cells were further stained with a drop of the DNA-specific dye DAPI and mounted in Permafluor (Lipshaw-Immunonon, Inc., Pittsburgh, PA). Ektachrome P1600 film was used when pictures were taken from a standard fluorescence microscope (Axiophot Photomicroscope; Zeiss).

Microinjection with Polyclonal Antibodies and mAbs. The polyclonal antibodies and mAbs for microinjection were prepared as described above. Cells were injected with antibodies at concentrations of 2 mg/ml in microinjection buffer [20 mM $NaHPO_4$ (pH 7.2), 0.1 mM EDTA, and 10% glycerol] using a microinjection apparatus (Eppendorf) as described previously (12).

Transfections. Three constructs were used in transfection assays: (*a*) CHPL-GFP, which was a derivative plasmid of a mammalian expression vector containing a myc-tagged mutant form of GFP (S65T; Ref. 13); (*b*) CHPL-GFP-TSG101, which contains GFP fused to the amino terminus of the full-length *TSG101* cDNA; and (*c*) NCF-TSG101, which contains the full-length *TSG101* cDNA with an amino-terminal FLAG tag (Kodak). For transfection, purified plasmid DNA was either mixed with Lipofectin reagent (Life Technologies, Inc., Grand Island, NY) or precipitated using standard calcium phosphate techniques and added to 1×10^6 cells. The mixtures were removed 18 h after transfection, and the cultures were refed with fresh medium. Living cells were observed under a fluorescence microscope at different time points or fixed at 48–72 h after transfection.

Results

Anti-TSG101 Antibodies Specifically Recognized a 46-kDa Protein in Human Cells. Mouse polyclonal antiserum raised against a GST-TSG101 fusion protein (amino acids 167–374) has been described previously (5). A panel of mAbs to TSG101 was generated from the same segment of TSG101 (see "Materials and Methods"). The specificity of the antibodies was validated by immunoblotting using unlabeled whole-cell lysates. The mouse polyclonal antibodies and mAbs were found to specifically recognize a 46-kDa band in T24 bladder carcinoma cell lysates (Fig. 1) and in lysates from several other cell lines (data not shown). One of the mAbs, 4A10, was used for additional studies.

Subcellular Localization of TSG101. T24 bladder carcinoma cells were chosen to examine the expression and subcellular localization of TSG101 during cell cycle progression, because these cells are conveniently arrested in G_0 by contact inhibition and display very high synchrony on replating at low density in fresh medium (11). T24 cells were fixed at different time points corresponding to each stage of the cell cycle and immunostained with specific anti-TSG101 antibodies. In G_1-phase cells, TSG101 showed predominantly cytoplasmic staining and weak staining in the nuclei (Fig. 2*A*, *panel 1*). On cell cycle progression to S phase, the intensity of nuclear staining for TSG101, coinciding with the DAPI staining, increased and became dominant in late S phase, possibly due to the increased translocation of TSG101 from the cytoplasm to the nucleus (Fig. 2*A*, *panel 2*). In mitotic cells, the majority of TSG101 was present in areas surround-

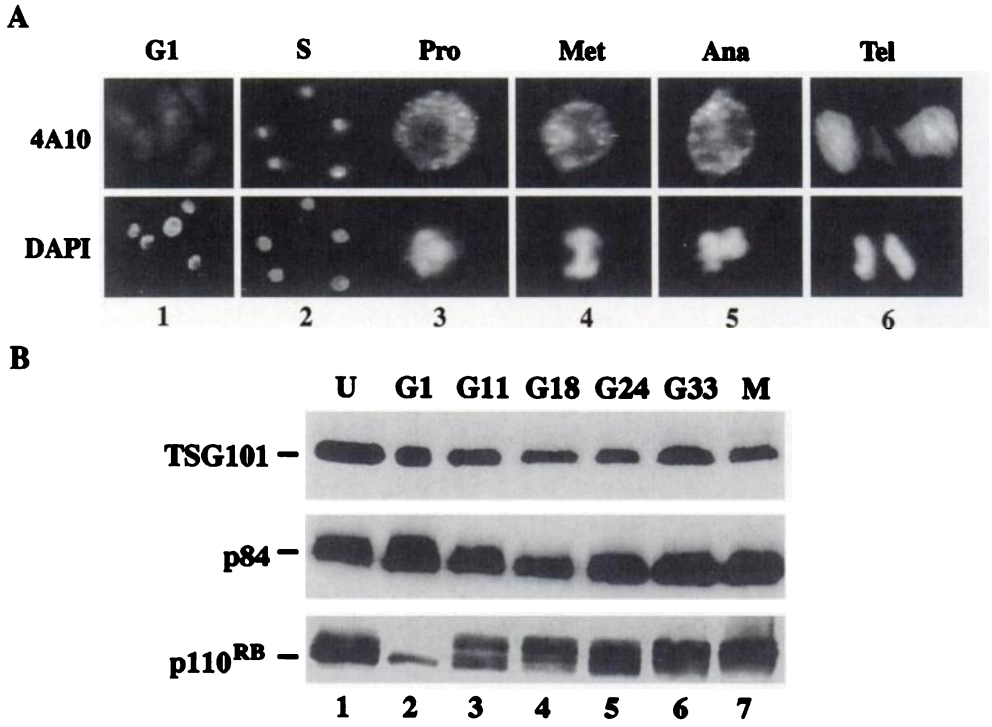

Fig. 2. Subcellular localization and expression of TSG101 during cell cycle progression. *A*, immunostaining of TSG101 during different phases of the cell cycle. *Panels 1*, T24 cells fixed in late G_1 phase show speckled cytoplasmic staining and weak staining in the nuclei (original magnification, ×500); *panels 2*, cells in S phase show cytoplasmic staining and strong nuclear staining; *panels 3–6*, cells in G_2-prophase, metaphase, anaphase, and telophase, respectively, (higher magnification, ×1250) showing strong staining surrounding the chromosomes and weak chromosome staining. *Upper panels* were stained with anti-TSG101 mAb 4A10, and *lower panels* were stained with DAPI. *B*, TSG101 expression is constant during the cell cycle. T24 cells were either unsynchronized (*Lane 1*) or synchronized at G_0-G_1 and released and then harvested at various time points (*Lanes 2–7*). Hypophosphorylated retinoblastoma protein ($p110^{RB}$) and various phosphorylated forms ($pp110^{RB}$) marked stages of the cell cycle: G_1, *Lanes 2* and *3*; G_1-S-phase boundary, *Lane 4*; S phase, *Lane 5*; G_2-M phase, *Lane 6*; and nocodazole-arrested cells, *Lane 7*. p84 served as an internal control for protein loading.

ing the chromosomes, although weak chromosomal staining was also observed (Fig. 2*A*, *panels 3–6*). Similar staining patterns were observed using both mouse polyclonal antibodies and other mAbs (data not shown). The staining pattern of TSG101 was distinct from the spindle staining or other microtubule staining when cells were costained with anti-tubulin antibodies (data not shown).

The amount of TSG101 protein in cells is nearly constant during cell cycle progression. Using the above-described synchronized T24 cells, cell lysates were collected and analyzed by straight Western blot analysis with anti-TSG101 mAb 4A10. Cell cycle stages were verified by cell cycle-dependent phosphorylation of the retinoblastoma protein (14). As shown in Fig. 2*B*, the amount of TSG101 in cells during different stages was constant. This was also the case for nuclear matrix protein p84, which was stable throughout the cell cycle (15) when the same amount of cell lysate was analyzed. Similar results were observed using both mouse polyclonal antibodies and other mAbs (data not shown). Because the nuclear staining of TSG101 was found to increase significantly in cells in late S phase, it seems likely that the cytoplasmic TSG101 had translocalized to the nucleus.

Microinjection of Anti-TSG101 Antibodies Arrests Cell Cycle Progression. To test directly whether TSG101 is important for cell cycle progression, we prepared anti-TSG101-specific mAb 4A10 for microinjection into cultured cells to inactivate endogenous TSG101. As described above, this mAb recognized the same 46-kDa protein as the polyclonal anti-TSG101 serum in either straight immunoblotting or immunoprecipitation assays. T24 cells synchronized in G_1 or S phase were microinjected with mAb 4A10. When all cells should have completed mitosis, the majority of cells injected with mAb 4A10 remained undivided (Fig. 3, *c* and *d*). Uninjected cells and those injected with the control antibody (total murine IgG) underwent normal cell division (Fig. 3, *a* and *b*; Table 1). Similar results were also obtained when mouse polyclonal antibodies were used for microinjection (Table 1; data not shown). Taken together, these results suggest that the inactivation of endogenous TSG101 by the injected antibodies may perturb cell cycle progression at either the G_1-S-phase or G_2-M-phase transition. This observation is compatible with the previously described result that showed that inactivation of TSG101 by antisense RNA causes cellular transformation.

Overexpression of TSG101 Leads Cell to Death. The other strategy to disturb the overall balance of the TSG101 protein in cells is to overexpress exogenous proteins. A construct containing the full-length *TSG101* cDNA fused to GFP under the regulation of the cytomegalovirus immediate early gene promoter was designed for this purpose. The plasmid containing GFP alone served as a control. The transfection of these constructs into human osteosarcoma cell line Saos-2 resulted in expression of the GFPs, which could be detected under the fluorescence microscope. The expression of GFP or GFP-TSG101 was verified by immunoprecipitation of the transfected cell lysates with anti-myc mAb or anti-TSG101 mAb followed by Western blot analysis with anti-GFP mAb (Fig. 4*A*; data not shown). Initially, many scattered small green dots were seen in the cytoplasm of divided cells expressing the GFP-TSG101 fusion protein (Fig. 4*B*, *panel c*). After 60 h, the green dots in most of the transfected cells were found to accumulate around the nuclei, and some dots were in the nuclei (Fig. 4*B*, *panels e* and *g*). In cells with a high level of GFP-TSG101, the fluorescence was colocalized with degraded nuclear debris containing chromosome DNA that could be stained by DAPI (Fig. 4*B*, *panels i–k*). The majority of these cells did not survive. Very few low-fluorescence cells remained alive 96 h after transfection (data not shown). This observation suggests that the expression of low levels of TSG101 protein may not significantly disturb cell division; however, overexpression of TSG101 induces cell death. Similar results were also observed after transfection with the FLAG-tagged TSG101 fusion protein expression construct.[4] Cells transfected with GFP alone had both nuclear and cytoplasmic fluorescence and divided normally (Fig. 4*B*, *panels a* and *b*). These results suggest that disturbing the delicate level of TSG101 protein in cells is detrimental to their growth.

Table 1 *T24 cells microinjected with mouse IgG, mAb 4A10, or mouse polyclonal antibody*

Experiment no.	Injection time	Injected Ab[a]	Divided cells	Undivided cells	Total
1	G_1	Mouse IgG	126	38	164
	G_1	Polyclonal Ab	1	80	81
	S	Mouse IgG	171	28	199
	S	Polyclonal Ab	26	132	158
2	G_1	Mouse IgG	156	45	201
	G_1	4A10	23	133	156
	S	Mouse IgG	114	37	151
	S	4A10	13	146	159
3	G_1	Mouse IgG	73	18	91
	G_1	4A10	11	79	90
	S	Mouse IgG	68	14	82
	S	4A10	1	76	77

[a] Ab, antibody.

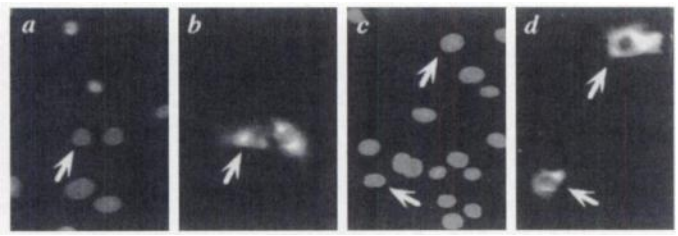

Fig. 3. Microinjection of anti-TSG101 results in cell cycle arrest. *A*, T24 cells were released from density arrest and allowed to proceed through the cell cycle. Twelve or 24 h after release, the majority of the cells were in G_1 or S phase, respectively, at which time they were microinjected with either nonspecific mouse IgG (*a* and *b*) or mAb 4A10 (*c* and *d*). Fifty h after release, after they should have passed through mitosis, cells were fixed and analyzed by indirect immunofluorescence staining. *a* and *c*, DAPI fluorescence; *b* and *d*, staining with antimouse IgG antibodies. *Arrowheads*, the daughters of successfully microinjected cells.

Discussion

Functional inactivation of tsg101 in murine NIH3T3 fibroblasts by antisense RNA using the random homozygous knockout procedure led to neoplastic transformation (1). The high frequency of microtubule and mitotic spindle anomalies seen in these cells suggests that TSG101 may have a crucial role in M-phase progression (16). Inactivation of TSG101 in mammalian cells by antibody microinjection causes cell cycle arrest. This result is consistent with the notion that the TSG101 protein seems to be important for cell growth. Similarly, disturbance of the delicate level of TSG101 protein in cells by overexpression of a TSG101 fusion protein led to cell death. Based on structural information, TSG101 mimics a group of ubiquitin-conjugating enzymes in its amino-terminal region, but not at the active site for ubiquitin conjugation. This opens the possibility that TSG101 may serve as a dominant negative regulator of some ubiquitin-conjugating enzymes (6). Ubiquitin-conjugating enzymes have been shown to be important in several stages of cell cycle control (17). The ubiquitin-dependent degradation of anaphase inhibitor Pds1p and cyclin B is required for metaphase-anaphase progression and telophase-G_1 progression, respectively (18–20). Disruption of the overall balance of TSG101 protein would perturb the regulation of this proteolytic pathway. However, the precise molecular basis remains to be tested biochemically.

TSG101 localizes in the cytoplasm during the G_1 phase of the cell cycle and translocates to the nucleus during late S phase. At M phase,

[4] Unpublished observations.

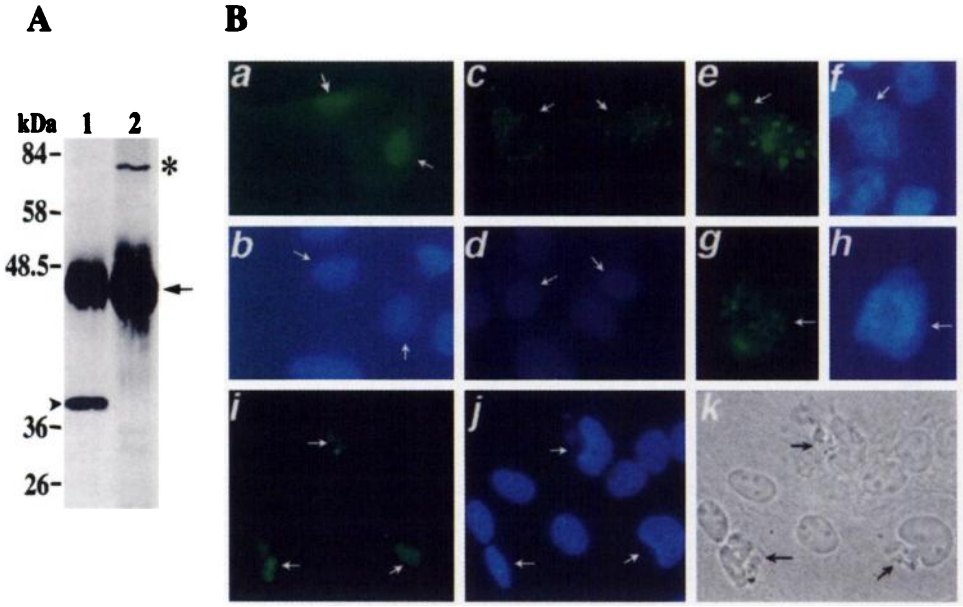

Fig. 4. Ectopic expression of TSG101 leads to cell death. *A*, detection of GFP and GFP-TSG101 fusion proteins in transfected Saos-2 cells. After transient transfection, the expression of GFP fusion proteins was determined by immunoprecipitation with an anti-myc-9E10 antibody, followed by blotting with anti-GFP mAb. *Asterisk*, GFP-TSG101 (*Lane 2*) fusion protein; *arrowhead*, GFP (*Lane 1*); *arrow*, the heavy chain of immunoglobulin (*Lanes 1* and *2*). *B*, localization of GFP and GFP-TSG101 fusion proteins in Saos-2 cells. DAPI fluorescence (*b, d, f, h,* and *j*) and GFP autofluorescence (*a, c, e, g,* and *i*) were observed under a fluorescence microscope. Phase contrast is also shown (*k*). Initially, many scattered small *green dots* were seen in the cytoplasm of divided cells expressing the GFP-TSG101 fusion protein (*c*). After 60 h, the green dots in most of the transfected cells were found to accumulate around the nuclei, and some dots were in the nuclei (*e* and *g*). In cells with a high level of GFP-TSG101, the fluorescence was colocalized with nuclear debris containing chromosome DNA that could be stained by DAPI (*i–k*). The cells transfected with GFP alone have both nuclear and cytoplasmic fluorescence and divided normally (*a* and *b*). The *arrows* indicate nuclei containing fluorescent proteins.

it surrounds the newly condensed chromosome. The amount of TSG101 protein throughout the cell cycle is constant, suggesting that TSG101 may be regulated by a posttranslational mechanism. Because TSG101 may be important for cell division, its nuclear transportation could be an important event for its function. However, the mechanism of TSG101 protein translocation into the nucleus remains unclear. A traditional nuclear localization signal is missing in TSG101. Other nuclear transportation mechanisms may be involved in this process. A conventional modification such as phosphorylation of the TSG101 protein does not seem to be the factor influencing this process in our initial studies (data not shown). Alternatively, it will be interesting to test whether small ubiquitin-related modifier 1 conjugation, a novel regulation of the cytoplasmic-nuclear transportation (21–23), is important for TSG101 translocation.

Recently, Xie *et al.* (16) reported that TSG101 is present in both the cytoplasm and the nucleus, predominantly in the nucleus and Golgi complex during G_1, and is dispersed more generally throughout the cytoplasm when cells reach late S phase. Furthermore, the protein is localized in mitotic apparatus such as the spindle during mitosis (16). Our results did not reveal any obvious staining of the Golgi complex in the interphase, and staining of the microtubule apparatus during mitosis was also not observed. This discrepancy could be due to the antibodies used. The rabbit anti-TSG101 antibodies detected more than one species of protein by straight Western blot analysis (16); however, the mouse polyclonal antibodies or mAbs described here recognized only one protein by Western blot analysis. It is possible that different cell preparations for staining may also be critical with regard to this discrepancy.

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