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TRAM2 Protein Interacts with Endoplasmic Reticulum Ca^{2+} Pump Serca2b and Is Necessary for Collagen Type I Synthesis

Branko Stefanovic,[1]* Lela Stefanovic,[1] Bernd Schnabl,[2] Ramon Bataller,[3] and David A. Brenner[3,4]

Department of Biomedical Science, College of Medicine, Florida State University, Tallahassee, Florida[1]; Department of Internal Medicine I, University of Regensburg, Regensburg, Germany[2]; Departments of Medicine, Biochemistry, and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina[3]; and Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York 10032-3784[4]

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Cotranslational insertion of type I collagen chains into the lumen of the endoplasmic reticulum (ER) and their subsequent folding into a heterotrimeric helix is a complex process which requires coordinated action of the translation machinery, components of translocons, molecular chaperones, and modifying enzymes. Here we describe a role for the protein TRAM2 in collagen type I expression in hepatic stellate cells (HSCs) and fibroblasts. Activated HSCs are collagen-producing cells in the fibrotic liver. Quiescent HSCs produce trace amounts of type I collagen, while upon activation collagen synthesis increases 50- to 70-fold. Likewise, expression of TRAM2 dramatically increases in activated HSCs. TRAM2 shares 53% amino acid identity with the protein TRAM, which is a component of the translocon. However, TRAM2 has a C terminus with only a 15% identity. The C-terminal part of TRAM2 interacts with the Ca^{2+} pump of the ER, SERCA2b, as demonstrated in a *Saccharomyces cerevisiae* two-hybrid screen and by immunoprecipitations in human cells. TRAM2 also coprecipitates with anticollagen antibody, suggesting that these two proteins interact. Deletion of the C-terminal part of TRAM2 inhibits type I collagen synthesis during activation of HSCs. The pharmacological inhibitor of SERCA2b, thapsigargin, has a similar effect. Depletion of ER Ca^{2+} with thapsigargin results in inhibition of triple helical collagen folding and increased intracellular degradation. We propose that TRAM2, as a part of the translocon, is required for the biosynthesis of type I collagen by coupling the activity of SERCA2b with the activity of the translocon. This coupling may increase the local Ca^{2+} concentration at the site of collagen synthesis, and a high Ca^{2+} concentration may be necessary for the function of molecular chaperones involved in collagen folding.

Cirrhosis is characterized by the accumulation of extracellular matrix proteins in the liver, including type I collagen (3, 43). Hepatic stellate cells (HSCs, also named Ito cells, lipocytes, or fat-storing cells) are the major cell type responsible for collagen synthesis in the cirrhotic liver (16, 33). In a normal liver, quiescent HSCs store vitamin A (26) but express only trace amounts of type I collagen (46). Upon a fibrogenic stimulus, HSCs become activated, a process in which they lose retinoid droplets, proliferate, change morphologically into myofibroblasts, and increase their synthesis of extracellular matrix proteins (19, 30). Culturing quiescent HSCs on plastic causes activation similar to that seen in liver fibrosis in vivo, including the accumulation of COL1A1 mRNA and synthesis of type I procollagen (20, 30). COL1A1 mRNA increases 50- to 70-fold in activated HSCs compared to quiescent HSCs. Procollagen α1(I) [pro-α1(I)] is undetectable by Western blotting in quiescent HSCs, and its level starts to increase after 4 days in culture (unpublished result). After translation initiation, individual pro-α chains are cotranslationally inserted into the endoplasmic reticulum (ER). Insertion is driven by the ribosome engaged in translation elongation and attached to the translocon. The translocon is a gated channel through the ER membrane and is composed of Sec61α, Sec61β, Sec61γ, and the translocation-associated membrane protein (TRAM). Proteins associated with the translocon that are not integral components include signal peptidase, oligosaccharyltransferase, BiP, ribosome receptors, and the signal recognition particle receptor (28). During cotranslational insertion, pro-α chains undergo hydroxylation of prolyl and lysyl residues, glycosylation of hydroxylysyl residues, attachment of N-linked oligosaccharide in the C-terminal propeptide, and intrachain disulfide bond formation. Following interchain disulfide cross-linking, two pro-α1(I) chains and one pro-α2(I) chain are folded into a triple helix (29, 31, 54). There is evidence that procollagen chains initiate assembly into the trimer while they are still associated with ribosomes on the membrane of the ER (5, 8, 53). Modifications of individual chains and their assembly into a triple helix seem to be in kinetic equilibrium, because mutations which affect the rate of assembly result in hypermodifications of the chains (4, 18). The assembly process is facilitated by the actions of several molecular chaperones. Protein disulfide isomerase (PDI) is a subunit of prolyl hydroxylase that associates as a monomer with nascent pro-α chains before incorporation into the triple helix (7, 56). It has been postulated that PDI prevents misfolding of individual chains until they assemble into the trimer (6). Another molecular chaperone, BIP, has been reported to bind misfolded chains and target them for degradation (11). While PDI and BIP are ubiquitous chaperones, HSP 47 is chaperone specific for various collagens (37). HSP 47 seems to associate with prefolded procollagen triple helices, preventing their aggregation and facilitating their ex-

* Corresponding author. Mailing address: Department of Biomedical Science, Florida State University College of Medicine, Tallahassee, FL 32306. Phone: (850) 644-7600. Fax: (850) 644-8924. E-mail: branko.stefanovic@med.fsu.edu.

port into the Golgi complex (51, 52). HSP 47-deficient mice die at embryonic day 11 with abnormalities in type I and type IV collagen (36).

Molecular chaperones require Ca^{2+} for function, and some Ca^{2+} storage proteins in the ER are chaperones themselves (2, 15, 34, 35). Depletion of ER Ca^{2+} stores leads to the unfolded protein response (UPR) of the cell (22, 41). The main Ca^{2+} pump of HSCs and fibroblasts, which maintains a high Ca^{2+} concentration within the ER, is sarcoplasmic-ER Ca^{2+}-ATPase type 2b (Serca2b) (10, 27, 57). Serca2b is a ubiquitously expressed transmembrane protein of the ER (9), but its relationship with the translocon is unknown.

Here we report that TRAM2, a homolog of the TRAM protein, is upregulated during activation of HSCs and that the C-terminal part of TRAM2, which has a unique sequence, interacts with Serca2b. TRAM2 without the C terminus or treatment with pharmacological inhibitors of Serca2b inhibits procollagen synthesis in HSCs. We suggest that during activation of HSCs TRAM2 is required for recruitment of Serca2b to the translocon and that this couples procollagen synthesis and a Ca^{2+}-dependent function of molecular chaperones. This identifies TRAM2 as one of the key components required for excessive collagen synthesis by activated HSCs.

MATERIALS AND METHODS

Constructs. Adenovirus for expression of full-size human TRAM2 was constructed by cloning the TRAM2 reverse transcriptase PCR (RT-PCR) product made by reverse transcription of total RNA from activated human HSCs and amplified with the primers 5′-ATGGCTTTCCGCAGGAGGACG and 3′-GGGAGACTTGAGTTTCTTAGTCCG. The band of 1.1 kb was eluted from the gel and reamplified with a new set of primers in which the 5′ primer contained a *Sma*I site and the 3′ primer contained a *Sal*I site. This second PCR product was cloned into *Sma*I-*Sal*I sites of pGEM3 (Promega). Sequencing of the full insert confirmed the identity with the published sequence of the KIA0057 gene (GenBank accession number NM_012288) and no PCR errors. From this clone a *Kpn*I-*Sal*I fragment was recloned into *Kpn*I-*Xho*I sites of pCDNA3 vector (Invitrogen) for mammalian expression (H-TRAM2/pCDNA3). From the H-TRAM2/pCDNA3 clone, the full-size TRAM2 was recloned into *Kpn*I-*Xba*I sites of pAdCMV-Track vector (TRAM2/Track). TRAM2 truncated by 66 amino acids at the C terminus (TRAM2ΔC/Track) was constructed by removing a *Stu*I-*Eco*RV fragment of TRAM2/Track and religating the vector. A double-stranded oligonucleotide encoding two hemagglutinin (HA) tags was cloned in frame at the N terminus into both TRAM2/Track and TRAM2ΔC/Track clones. Adenoviruses expressing these tagged proteins were constructed by recombination with pAD-Easy DNA in *Escherichia coli* and packaged into viral particles by transfecting into 293 cells, as described previously (23). Control virus expressed a short nonfunctional RNA instead of a protein (mutant molecular decoy) (44). All viral vectors contained an additional independent transcription unit expressing green fluorescent protein (GFP), which served as a marker for viral infection.

Mouse TRAM2 was isolated by RT-PCR using total mouse liver RNA as the source of RNA and single-step PCR amplification with the primers 5′-ATGGC TTTCCGTAGGAGGAC and 3′-GGGAGACTTGAGTTTCTTAATC, followed by cloning of a 1.1-kb PCR product into PCR2.1TOPO vector (Stratagene). From this clone, a *Pst*I (blunt)-*Bam*HI fragment encoding the C-terminal 145 amino acids of mouse TRAM2 was cloned into *Sma*I-*Bam*HI sites of the *Saccharomyces cerevisiae* two-hybrid vector pGBT-9 as bait. This bait was used to screen an NIH 3T3 two-hybrid library (Clontech), using standard protocols for two-hybrid screening (Clontech). Probes for an RNase protection assay have been described before (46). Full-size human Serca2b cloned in the pCDNA3.1 vector was a kind gift of J. Lytton and was tagged with FLAG tag at the N terminus by cloning a double-stranded oligonucleotide with the FLAG sequence in frame at the 5′ end of the coding sequence.

Cell culture and viral infections. Rat HSCs were isolated according to published procedures (20). Total RNA was isolated either immediately after isolation (quiescent HSCs) or after 7 days of culturing (activated HSCs) (13). For expression studies, at day 2 after isolation the cells were infected at a multiplicity of infection of 500 with adenoviruses expressing TRAM2, TRAM2ΔC, or control virus and cultured for an additional 5 days. Cellular proteins and media were then collected and analyzed by Western blotting as described elsewhere (44, 47). Human HSCs were isolated by perfusion of human liver explants with collagenase and pronase as described previously (42). Cells were cultured for the indicated time periods, and total RNA was extracted. Fibroblasts were grown in 10% fetal calf serum and, where indicated, thapsigargin (Sigma) was added to cell medium at 200 nM and calmidazolium Cl (Sigma) was added at 10 μM. MG132 (Sigma) was added at 100 μM, lactacystin (Sigma) was added at 50 μM, and puromycin (Sigma) was used at 30 μM for 2 h. In collagen secretion experiments, the unhydrolyzable analog of ascorbic acid, AA-2P (Waco), was added at 10 mM.

RNA and protein analysis. Rat TRAM2 expression was estimated by RT-PCR of 100 ng of total RNA or 20 ng of poly(A)$^+$ RNA, isolated from quiescent or activated HSCs, using a TthRT-PCR kit (Perkin-Elmer) in the presence of [^{32}P]dCTP, as described previously (47). The primers were 5′-TTGGCTTCGG ATTGGCTCGG and 3′-GCTGGGAGTGGATGAAACGC, which amplify a 145-nt product; 20 cycles were used for poly(A)$^+$ RNA, and 30 cycles for total RNA were used. The annealing and extension temperature was 55°C. Total RNA from human HSCs was analyzed as above using the primers 5′-TTGGCTTTG GACTGGCTCGC and 3′-GCTGGGAGTGGATGAAGCGC, which give a 143-nt product. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control amplification was done as described before (47). PCR products were resolved on 6% denaturing gels, and the bands were excised, reamplified with the same primers, and sequenced to confirm the identity of the products. TRAM and Serca2b expression was analyzed by RT-PCR as for TRAM2 using the following primers: for TRAM, 5′-GAGCCATGAATTCGTGCTGC and 3′-GCTGATTC AGCAGCTTGTTC; for Serca2b, 5′-CAAGGTGTATCGACAGGACAG and 3′-CCACTGGTCAACTCTCAGAG. The annealing and extension temperature was 50°C. RNase protection assays for collagen α1(I) mRNA were done as described previously (45, 46).

Western blotting assays were done with 50 μg of cellular proteins, as described elsewhere (44, 47). When cellular medium was analyzed, equivalent amounts of medium were used without prior concentration. The anticollagen antibody (600-401-103; Rockland) has been characterized in previous papers (44, 47). Anticalnexin antibody (C45520; BD Biosciences) and antifibronectin antibody (F14420; BD Biosciences) were used as recommended by the manufacturer.

Immunoprecipitations. Expression vector (pCDNA3.1) encoding FLAG-tagged human SERCA2b was transfected into human fibroblasts, and two stably transfected cell lines were developed by selection with G418 for 2 months. Expression was verified by Western blotting. Three p150 dishes of one cell line were infected with adenoviruses expressing HA-tagged human TRAM2 and HA-tagged human TRAM2ΔC. Two days after infection, cell lysate was prepared by homogenizing the cells in phosphate-buffered saline containing 0.5% NP-40 and, after removal of nuclei by centrifugation, 1 mg of clear lysate was incubated with either 10 μl of anti-HA monoclonal antibody (HA-7; Sigma), 10 μl of anti-FLAG monoclonal antibody (M2; Sigma), 10 μl of antitubulin monoclonal antibody (MSA78G; Accurate), or 10 μl of anticollagen polyclonal antibody (600-401-103; Rockland) for 1 h at 4°C. A 50-μl volume of prewashed protein A/G PLUS-agarose (Santa Cruz Biotechnology) was added to each tube, and incubation continued for 1 h. After washing three times in lysis buffer, samples were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer and analyzed by Western blotting.

Measurement of intracellular Ca^{2+}. Cells cultured in 24-well plates were loaded with the Ca^{2+}-sensitive dye Fluo-4 (10 μM; Molecular Probes, Eugene, Oreg.) for 20 min at 37°C. Cells were then rinsed twice with Dulbecco's modified Eagle's medium and stimulated with dimethyl sulfoxide (DMSO) or thapsigargin. Changes in intracellular Ca^{2+} formation were estimated by measuring cell fluorescence at 485/535 nm using a multiwell fluorescence scanner (CytoFluor 2300; Millipore, Bedford, Mass.).

RESULTS

TRAM2 is expressed in activated HSCs. To identify genes differentially expressed in HSCs, we compared gene expression profiles of activated HSCs and whole liver by using DNA microarrays (unpublished data). One gene that was expressed at a 3.3-fold higher level in HSCs than in whole liver was a homolog of the TRAM protein. This protein has been sequenced as a part of human cDNA sequencing effort and named KIAA 0057 (39); here we have named this protein

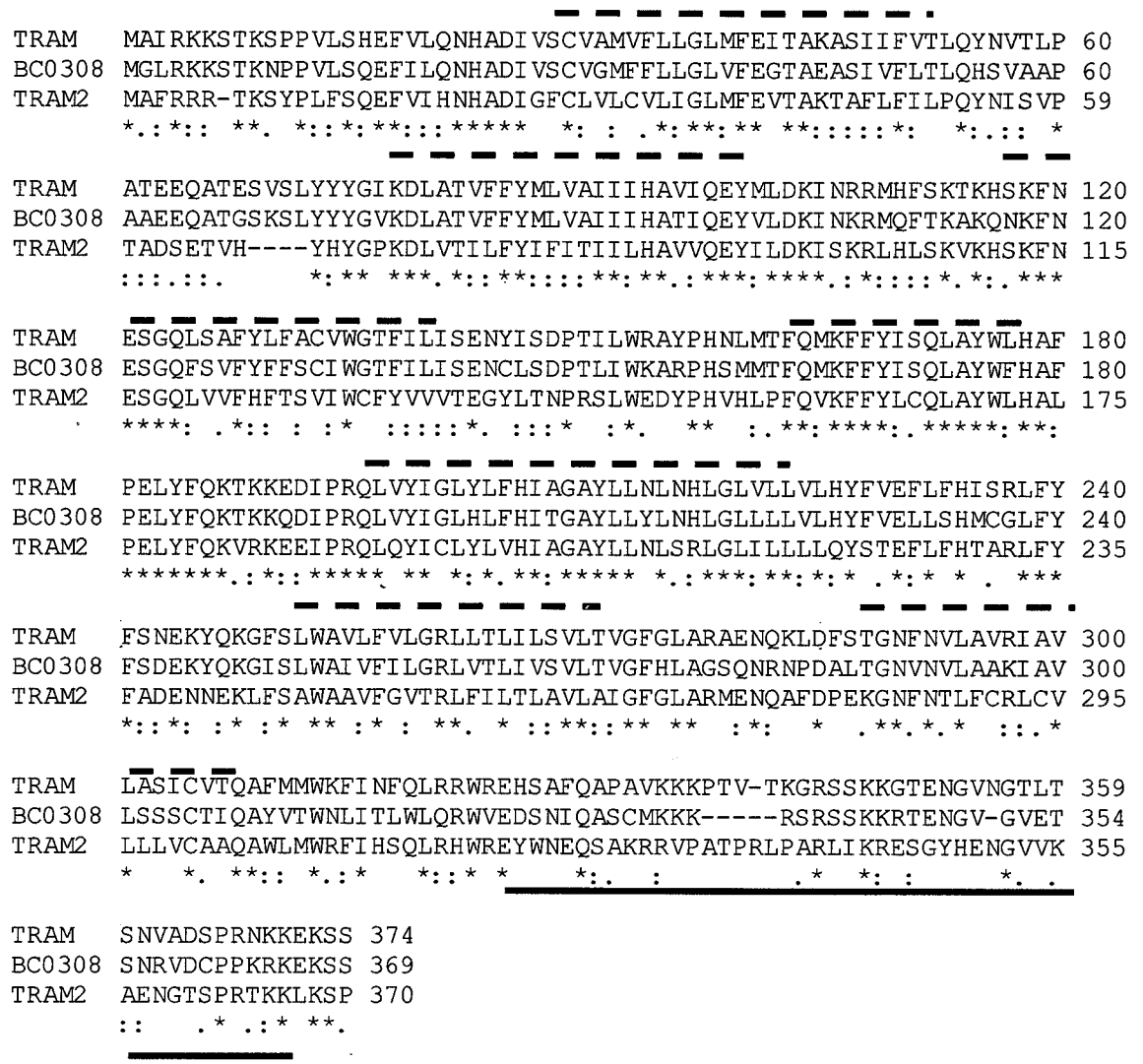


FIG. 1. Sequence comparison between human TRAM, TRAM2, and BC030831. The amino acid sequences of TRAM (accession no. S30034), TRAM2 (KIAA0057; accession no. D31762), and TRAM-like protein (accession no. BC030831) were aligned using the ClustalW program. Identities are indicated as asterisks under the sequence, conserved residues in at least two proteins are shown as double dots, and conservative substitutions are indicated by single dots. Putative transmembrane regions are overlined with dotted lines, and the most divergent C-terminal domain is underlined with a solid line.

TRAM2. Since extracellular matrix proteins, primarily collagen type I, are secreted from HSCs and not from hepatocytes, and since TRAM is an integral part of the translocon, we surmised that TRAM2 may be involved in collagen secretion from HSCs. Figure 1 shows the homology between human TRAM and TRAM2 proteins, as well as with another member of the TRAM superfamily with accession no. BC030831. Overall, there was 53% amino acid identity between TRAM and TRAM2. TRAM has seven putative transmembrane regions (Fig. 1), and the same topology can be predicted for TRAM2. Transmembrane segments show the greatest similarity between the two proteins. The most divergent part is the C terminus, which shows only 15% identity within a stretch of 40 amino acids (Fig. 1). This region of TRAM2 has no high homology to any other protein, as assessed by a BLAST program search (1). We cloned TRAM2 from the mouse, and it showed 92% homology to the human ortholog, including the C-terminal region (data not shown). There is yet another homolog of human TRAM in GenBank: it is named "similar to TRAM," with accession number BC030831. The overall identity of this homolog to TRAM is 71% and its C-terminal domain is 50% identical to TRAM, suggesting that these two proteins are more related than TRAM and TRAM2 (Fig. 1).

Next, we validated expression of TRAM2 during culture activation of rat HSCs by RT-PCR. Poly(A)$^+$ RNA (Fig. 2A, lanes 1 and 2) or total RNA (lanes 3 and 4) was extracted from quiescent rat HSCs and from fully activated HSCs after 7 days in culture. In both RNAs, there was about 20- to 30-fold upregulation of TRAM2 mRNA in activated HSCs compared to quiescent HSCs. Both PCR products that routinely resolved on this gel represented TRAM2, as confirmed by excision of the bands out of the gel and sequencing. They probably re-

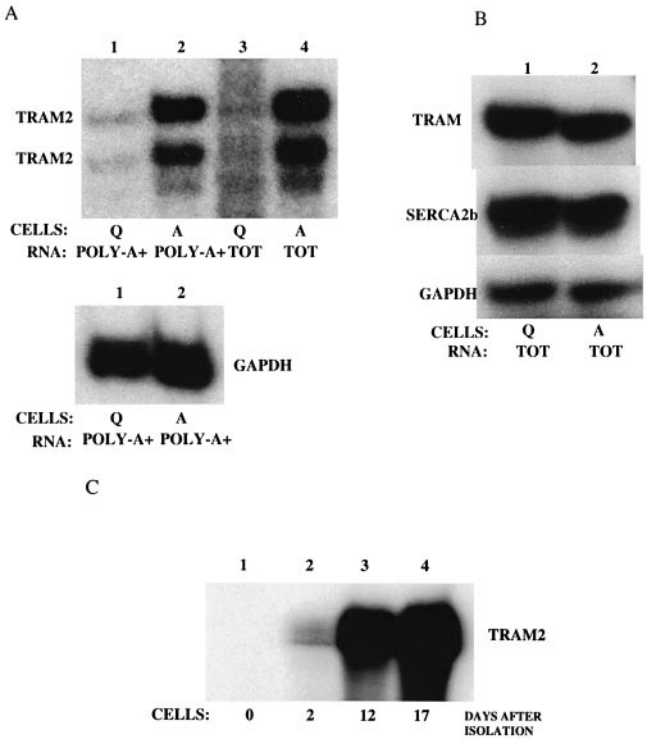


FIG. 2. TRAM2 mRNA is induced in activated HSCs. (A) Expression in rat HSCs. Shown are results of RT-PCR analysis of RNA isolated from fresh rat HSCs (Q) or from rat HSCs activated in culture for 7 days (A). Twenty nanograms of poly(A)$^+$ RNA (lanes 1 and 2) or 100 ng of total RNA (lanes 3 and 4) from two independent isolates of HSCs was subjected to RT-PCR analysis with primers specific for rat TRAM2. Poly(A)$^+$ RNA samples were also analyzed with primers specific to GAPDH (bottom panel). Migration of TRAM2-specific bands is indicated. Both bands represent the same PCR product, as confirmed by sequencing (see Results). (B) Expression of TRAM and Serca2b in rat HSCs. RT-PCR was done as described for panel A, with 100 ng of total RNA extracted from quiescent (Q) and activated (A) rat HSCs and with primers specific for TRAM (top panel), Serca2b (middle panel), or GAPDH (bottom panel). Migration of PCR products is indicated. GAPDH served as an internal control. (C) Expression in human HSCs. A 100-ng aliquot of total RNA from freshly isolated human HSCs (0, lane 1) or from human HSCs cultured for 2 days (lane 2), 12 days (lane 3), or 17 days (lane 4) was analyzed by RT-PCR with primers specific for human TRAM2. Identity of the bands was confirmed by sequencing and is indicated on the right.

sulted from denaturation of the double-stranded PCR product in the denaturing gel with differential migration of the two strands. So, total expression of TRAM2 was the sum of both bands. Equal loading for poly(A)$^+$ RNA was confirmed by analyzing GAPDH mRNA (bottom panel). Using RT-PCR, we also estimated expression of TRAM and Serca2b in quiescent and activated rat HSCs. The steady-state levels of TRAM and Serca2b mRNAs did not change during activation of HSCs (Fig. 2B). We also analyzed TRAM2 expression during culture activation of human HSCs (Fig. 2C). No TRAM2 mRNA could be detected in freshly isolated human HSCs (lane 1). At day 2 in culture, while the cells were still in a quiescent state, the mRNA was weakly expressed (lane 2); however, by days 12 and 17 (lanes 3 and 4) there was an approximately 20- to 50-fold increase in the steady-state level of TRAM2 mRNA compared to that at day 2. Thus, the result with human HSCs was similar to the result with rat HSCs. COL1A1 mRNA is increased 50- to 70-fold during culture activation of HSCs (46), and so expression levels of TRAM2 and COL1A1 mRNAs were parallel.

TRAM2 interacts with the Ca^{2+} pump of the ER Serca2b. Based on the homology of TRAM2 and TRAM, especially due to conservation of the transmembrane domains, it is likely that TRAM2 also functions as a part of the translocon. To provide insight into the function of the divergent C-terminal domain, we performed a yeast two-hybrid screen to identify proteins that interact with this domain of TRAM2 protein. As bait in this screen, we used the cDNA which encodes the 145 C-terminal amino acids of mouse TRAM2 and screened a library of mouse fibroblast cDNA as representative of collagen-producing cells. We screened 6 million clones and identified four independent clones that specifically interacted with TRAM2. They all encoded the C terminus of SERCA2b (57), another ER transmembrane protein. To confirm that the two proteins interact in human cells, we FLAG tagged human SERCA2b and stably expressed the protein in human fibroblasts and then infected these cells with adenovirus expressing HA-tagged human TRAM2 or TRAM2ΔC. Expression of the tagged proteins was determined by Western blotting (Fig. 3A, lane 1, B, lanes 1 and 3, and C, lane 1). When the cellular proteins were immunoprecipitated with anti-FLAG antibody and analyzed with anti-HA antibody, a strong signal was observed (Fig. 3A, lane 2). Antitubulin antibody gave only a weak background signal (lane 3), suggesting that there was a specific interaction between the two tagged proteins. When anticollagen antibody was used for immunoprecipitation, HA-tagged TRAM2 protein was also pulled down (lane 4). Similar results were obtained in the reverse experiment, when immunoprecipitation was done with anti-HA antibody and the samples were analyzed with anti-FLAG antibody (Fig. 3C, lane 2). In this experiment, however, anticollagen antibody did not pull down Serca2b protein (lane 3), suggesting that there was no interaction between procollagen α1(I) and Serca2b. When we expressed TRAM2ΔC and immunoprecipitated with anti-FLAG antibody, no TRAM2ΔC protein was coimmunoprecipitated with Serca2b (Fig. 3B, lane 2). This result suggests that the C terminus of TRAM2 interacts specifically with Serca2b and is consistent with yeast two-hybrid screening.

As additional controls for specificity of immunoprecipitations, two experiments were performed. First, immunoprecipitation was performed with anti-HA antibody and pulled down material was analyzed with anticalnexin antibody. Calnexin is an integral membrane protein of the ER. We could not detect any calnexin signal after immunoprecipitation with anti-HA antibody (Fig. 3D, lane 2), while this antibody strongly recognized calnexin in the initial sample (lane 1). This suggests that TRAM2 does not interact with calnexin. Second, we treated the cells with puromycin for 2 h to inhibit protein synthesis. During this time period there was no decrease in the steady-state level of pro-α1(I) or TRAM2, as analyzed by Western blotting (Fig. 3E, lanes 1 and 2). Then, we performed immunoprecipitation with anticollagen antibody and analyzed the amount of TRAM2 precipitated. As shown in Fig. 3E, lanes 3 and 4, there was less TRAM2 coprecipitated with procollagen in puromycin-treated cells. This indicates that there is a trans-

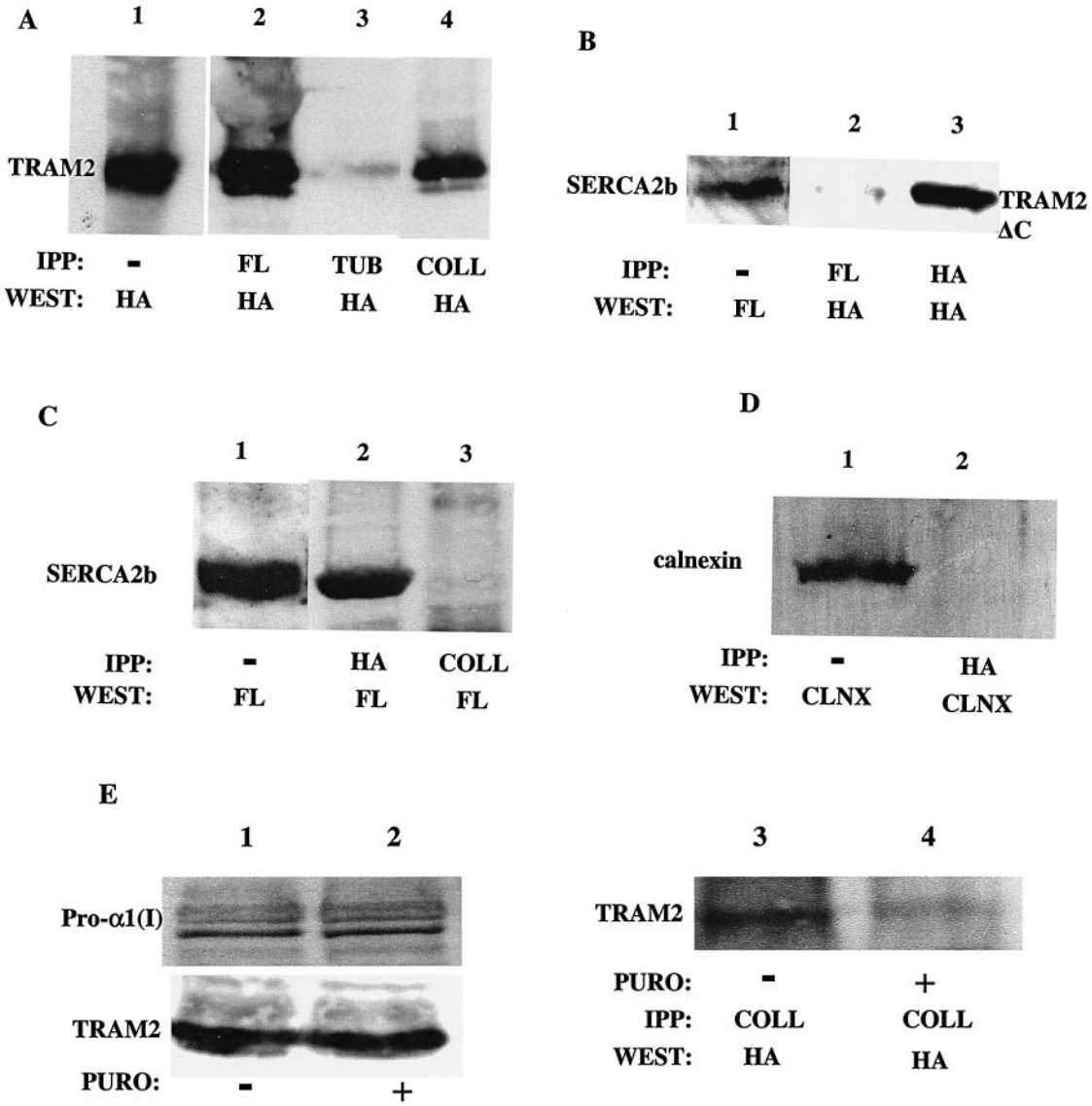


FIG. 3. TRAM2 and Serca2b proteins interact in human fibroblasts. (A) TRAM2 coprecipitates with Serca2b and with type I procollagen. FLAG-tagged human Serca2b protein and HA-tagged human TRAM2 protein were expressed in human fibroblasts. Immunoprecipitation (IPP) was done with 1 mg of cellular extract and using anti-FLAG antibody (lane 2; FL), antitubulin antibody (lane 3; TUB), or anticollagen antibody (lane 4; COLL). Western blotting (WEST) was done with the precipitated material using anti-HA antibody (HA). Lane 1 is Western blotting only, showing expression of HA-TRAM2 in 50 μg of cellular extract. (B) TRAM2ΔC does not interact with Serca2b. This experiment was done as described for that in panel A, except that HA-tagged TRAM2ΔC and FLAG-tagged Serca2b were coexpressed. Immunoprecipitation (IPP) was done with anti-FLAG antibody (lane 2) or anti-HA antibody (lane 3), and Western blotting (WEST) was done with the precipitated material using anti-HA antibody (lanes 2 and 3). Lane 1 is Western blotting only, showing expression of Serca2b in 50 μg of cell extract. (C) Serca2b coprecipitates with TRAM2, but not with procollagen. Immunoprecipitations were done with the same material as used for panel A, except that antibodies used for IPP were anti-HA (lane 2, HA) or anticollagen (lane 3, COLL). Western blotting was done with anti-FLAG antibody (FLAG). Lane 1 is Western blotting only, showing expression of FLAG-Serca2b in 50 μg of cellular extract. (D) Calnexin does not coprecipitate with TRAM2. Immunoprecipitations were done with the same material as used for panel A, except that the antibody used for IPP was anti-HA (lane 2, HA). Western blotting was done with anticalnexin antibody (CLNX). Lane 1 is Western blotting only, showing expression of calnexin in 50 μg of cellular extract. (E) Interaction of TRAM2 and procollagen is translation dependent. Cells were incubated with puromycin (+) or without (−) for 2 h, and extracts were analyzed by Western blotting for expression of procollagen and TRAM2 (lanes 1 and 2). The same samples were subjected to immunoprecipitation with anticollagen antibody (COLL) (lanes 3 and 4). Western blotting was done with anti-HA antibody (HA). The signal from coimmunoprecipitated TRAM2 protein in the absence (−) or presence (+) of puromycin is indicated as TRAM2.

lation-dependent interaction between pro-α1(I) and TRAM2, consistent with the interpretation that TRAM2 is involved in cotranslational translocation of pro-α(I) chains. From the yeast two-hybrid screen and immunoprecipitation experiments, we concluded that TRAM2 interacts with Serca2b and with pro-α1(I) chains.

TRAM2 with a C-terminal deletion inhibits collagen synthesis by HSCs. The functional significance of the interaction

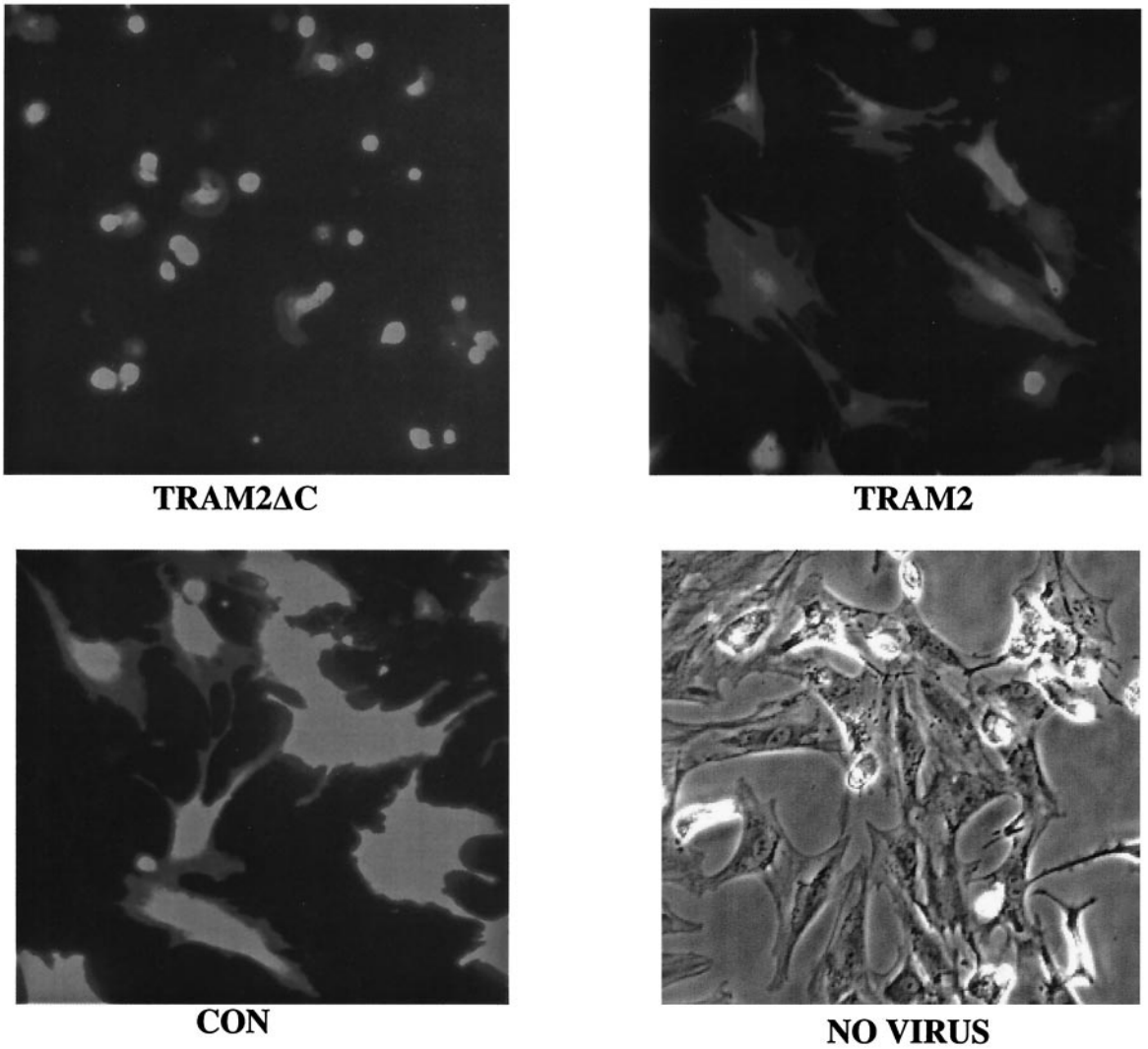


FIG. 4. Phenotype of rat HSCs expressing C-terminus-truncated TRAM2 (TRAM2ΔC). Rat HSCs were infected at day 2 after isolation with adenoviruses expressing TRAM2 with deletion of 66 amino acids at the C terminus (top left panel), expressing full-size human TRAM2 (top right panel), or expressing control virus (CON, bottom left panel). All viruses also expressed GFP as a marker of infection. After an additional 5 days (7 days after isolation), an image of the cells was taken under UV light so that only cells expressing the viruses were visible. In the bottom right panel are HSCs cultured without virus for the same time period. This image was taken under visible light.

between TRAM2 and Serca2b was addressed by overexpressing a C-terminal-truncated form of human TRAM2 during activation of HSCs. We deleted 66 amino acids from the C terminus of human TRAM2 (TRAM2ΔC) and expressed the protein at day 2 after isolation of rat HSCs, that is, before the onset of increased collagen synthesis (20). The cells were then cultured for an additional 5 days during which collagen synthesis was activated, to assess if TRAM2ΔC interfered with this process. Full-size human TRAM2 served as one control in these experiments to account for any effects of expression of the human protein in rat cells. Adenovirus not expressing a protein (control virus) served as another control for the effects of viral load on HSCs. Cell morphology after expression of TRAM2ΔC for 5 days during activation of HSCs, compared to cell morphology after expression of full-size TRAM2 and expression of the control virus, as well as to noninfected HSCs, is shown in Fig. 4. Green staining of the cells (in the nucleus and cytoplasm) resulted from expression of GFP, in addition to TRAM2 and TRAM2ΔC, by the corresponding viruses and served as a marker of viral infection. Thus, only cells which received the virus were visualized, and they represented more than 90% of total cells. Cells expressing TRAM2ΔC were smaller and rounded (Fig. 4, top left panel) compared to the typical stellate appearance of cells expressing full-size TRAM2 (top right panel). The latter were similar in morphology to cells expressing the control virus (bottom left panel) and to cells cultured without virus (bottom right panel). Therefore, expression of human TRAM2 did not have a visible effect on rat HSCs, but TRAM2ΔC drastically changed the morphology of HSCs.

Next, we measured procollagen protein in HSCs expressing TRAM2ΔC and full-size TRAM2 and compared it to cells

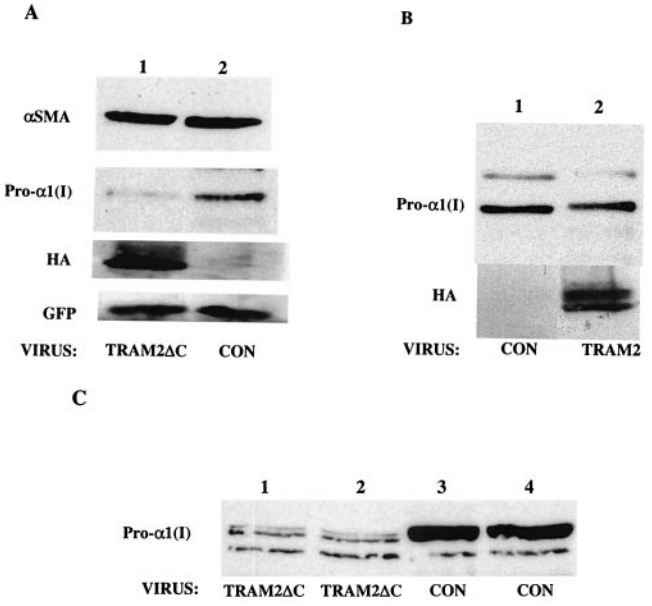
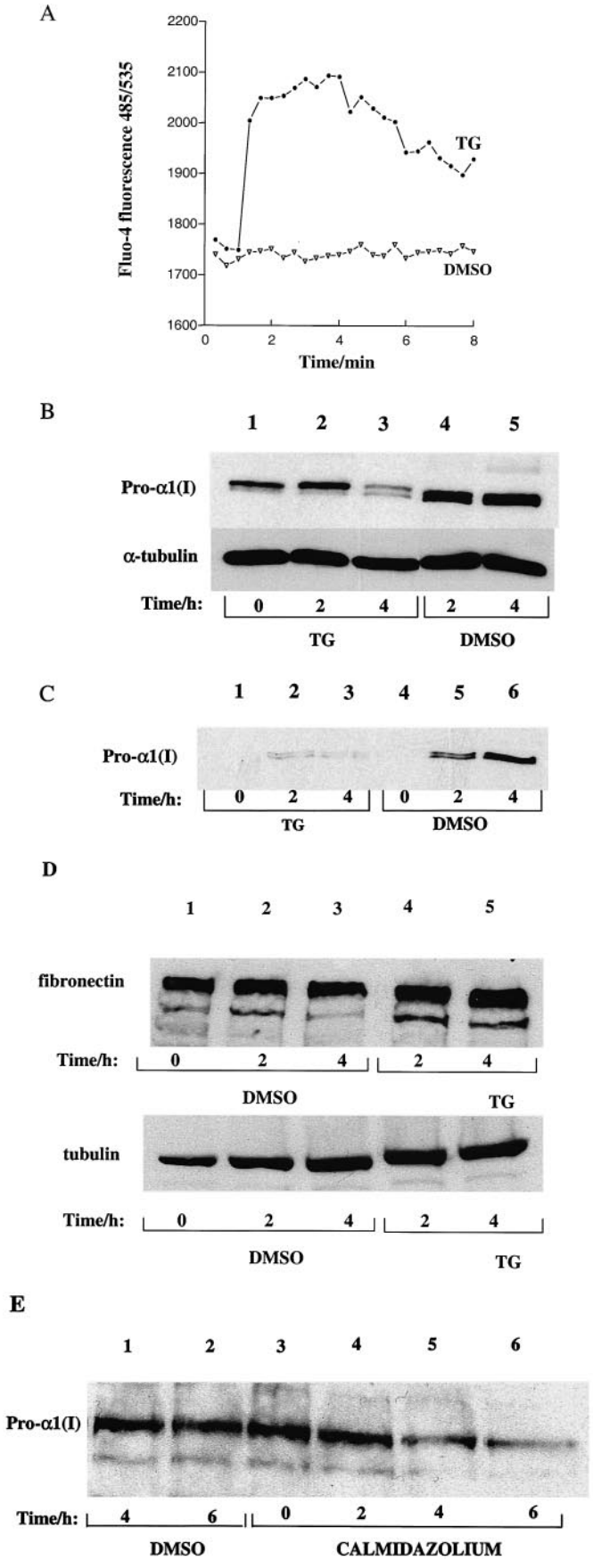


FIG. 5. TRAM2ΔC overexpression inhibits pro-α1(I) in rat HSCs. (A) Decrease in the cellular level of pro-α1(I). Rat HSCs were infected with adenovirus expressing TRAM2ΔC (lane 1) or control adenovirus (CON, lane 2) at day 2 after isolation and cultured for an additional 5 days. Western blots with 50 μg of cellular proteins were probed under reducing conditions with antibodies specific for α-SMA and pro-α1(I). Probing with anti-HA antibody (HA) was done as a control for TRAM2ΔC expression, and probing with anti-GFP antibody (GFP) served as a control for viral infection. (B) TRAM2 overexpression does not affect the level of pro-α1(I) in HSCs. This experiment was performed similar to that shown in panel A, except adenoviruses expressing full-size TRAM2 (TRAM2, lane 2) and control virus (CON, lane 1) were used. Western blotting was done with anticollagen antibody [pro-α1(I)] or anti-HA antibody (HA) to verify TRAM2 expression. (C) Two samples of cell medium of cells expressing TRAM2ΔC (lanes 1 and 2) or cells expressing control virus (lanes 3 and 4) were analyzed by Western blotting as described for panel A. Migration of the pro-α1(I) band is indicated.

expressing the control virus (Fig. 5). Cells expressing TRAM2ΔC accumulated less pro-α1(I) (Fig. 5A, lane 1) compared to cells infected with the control virus (lane 2). Expression of α-smooth muscle actin (α-SMA), another marker of HSC activation, was not affected, suggesting that TRAM2ΔC acts as an inhibitor of collagen synthesis but not as a general inhibitor of HSC activation. Expression of GFP was analyzed as a control for equal viral delivery, and expression of TRAM2ΔC was confirmed by Western blotting with anti-HA antibody. Procollagen accumulation in cells expressing full-length TRAM2 was similar to that in cells expressing the control virus (Fig. 5B, lanes 1 and 2). We also measured accumulation of procollagen in the cellular medium between days 6 and 7 (Fig. 5C) and found that cells overexpressing TRAM2ΔC accumulated about 10-fold less collagen in the medium than control cells during this 24 h. Based on these results, we concluded that expression of C-terminus-deleted TRAM2 severely impairs the ability of HSCs to accumulate and secrete collagen type I.

Depletion of ER Ca^{2+} stores by thapsigargin and calmidazolium Cl diminishes collagen synthesis. Since the TRAM2 C-terminal domain interacts with the Ca^{2+} pump of the ER, Serca2b, we wanted to know if collagen synthesis is dependent on a high Ca^{2+} concentration in the ER. Therefore, we inhibited Serca2b with thapsigargin or calmidazolium Cl. A control experiment to show that thapsigargin inhibits Serca2b is shown in Fig. 6A. At 1.5 min after addition of thapsigargin to fibroblasts, the cytoplasmic Ca^{2+} concentration increased sharply, indicating Ca^{2+} release from the ER. A decrease in cytoplasmic Ca^{2+} after 4 min was due to Ca^{2+} leakage into Ca^{2+}-free cell medium. Thus, thapsigargin depleted ER Ca^{2+} stores within minutes. Next, we measured procollagen synthesis by HSCs after addition of thapsigargin. As shown in Fig. 6B, the intracellular procollagen level did not change after 2 h (lane 2), while it decreased after 4 h of thapsigargin treatment (lane 3). The level of α-tubulin remained unchanged (bottom panel). We also measured the rate of procollagen secretion into cell medium. For that, we changed the medium just before addition of thapsigargin, and therefore there was no procollagen in the medium at time zero (Fig. 6C, lanes 1 and 4). Newly accumulated procollagen within 4 h was analyzed by Western blotting (Fig. 6C). Thapsigargin-treated cells did not secrete procollagen, because we did not detect its appearance in the medium after 2 h (lane 2) or after 4 h (lane 3). Control cells showed a continuous increase in extracellular procollagen during the same time period (lanes 5 and 6). This result suggests that type I procollagen secretion into the cellular medium was affected almost immediately after thapsigargin addition; however, the intracellular procollagen level decreased only after several hours (Fig. 6B). We also measured intracellular levels of another secreted protein, fibronectin. Fibronectin levels were unaffected by thapsigargin treatment for up to 4 h (Fig. 6D). Thus, the procollagen steady-state level is more sensitive to Ca^{2+} depletion than the level of fibronectin, suggesting more subtle regulation. To exclude possible side effects of thapsigargin, we treated the cells with another Serca2b inhibitor, calmidazolium Cl. Again, there was a decrease in the intracellular procollagen level detected at 4 h after Serca2b inhibition (Fig. 6E). From these experiments we concluded that maintaining ER Ca^{2+} concentrations is critical for collagen accumulation and secretion by HSCs.

ER Ca^{2+} is required for collagen protein assembly. To show that ER Ca^{2+} is necessary for collagen synthesis in other collagen-producing cells and to provide insight into a mechanism of this effect, we treated rat fibroblasts with thapsigargin. Figure 7A shows that the intracellular pro-α1(I) level was decreased after 6 h of thapsigargin treatment (lane 2), it remained low at 12 h, and it returned to 50% of the starting level by 24 h, indicating partial recovery. During this period of thapsigargin treatment, the COL1A1 mRNA steady-state level remained unchanged (Fig. 7B), suggesting that the decreased procollagen protein level resulted from either increased protein degradation or decreased mRNA translation. To distinguish between these two possibilities, we treated rat fibroblasts with thapsigargin for 6 h in the presence or absence of the proteosome inhibitor MG132 (Fig. 7C). Thapsigargin alone caused a decrease in pro-α1(I), as before (lane 2). MG132 alone did not affect the pro-α1(I) level (lane 3); however, MG132 was able to prevent the thapsigargin-induced procollagen decrease (lane 4). To corroborate this finding, we employed another proteosome inhibitor, lactacystin. Figure 7D shows that in the presence of lactacystin, thapsigargin was



unable to induce a decrease in the intracellular procollagen level (lane 4). Thus, inhibition of protein degradation rescued the cellular level of procollagen protein, suggesting that Ca^{2+} depletion accelerates intracellular procollagen degradation.

To assess why type I procollagen is susceptible to intracellular degradation in the absence of high ER Ca^{2+} concentration, we asked if procollagen polypeptides could be folded into disulfide-bonded multimers when ER Ca^{2+} stores are depleted. We treated cells with thapsigargin and performed Western blotting under nonreducing conditions. As shown in Fig. 7E, 6 h after thapsigargin addition the level of disulfide-bonded high-molecular-weight procollagen (DBC) decreased dramatically, with faster kinetics and to a greater extent than the level of monomers [pro-α1(I)] (compare lanes 1 and 2). DBC was almost undetectable 12 h after addition of thapsigargin, but the cells still contained about 30% of the initial level of monomers (lane 3). Therefore, we concluded that accumulation of disulfide-bonded intracellular procollagen is Ca^{2+} dependent and that disruption of formation of S-S-bonded multimers of pro-α chains precedes its accelerated intracellular degradation.

DISCUSSION

Type I procollagen is a heterotrimeric secreted protein composed of two pro-α1(I) polypeptides and one pro-α2(I) polypeptide (29, 54). These polypeptides are cotranslationally inserted through translocons into the lumen of the ER, where they undergo posttranslational modifications and folding into a triple helix. Folding of procollagen type I is facilitated by the action of molecular chaperones (7, 25, 31, 32, 37). Chaperones are important in maintaining the balance between the rate of translation, rate of modifications, and rate of incorporation of the chains into the triple helix (12, 51). In the human disease osteogenesis imperfecta, mutations that impair assembly of the

FIG. 6. Inhibition of Serca2b decreases pro-α1(I) synthesis. (A) Thapsigargin depletes Ca^{2+} from the ER. A 200 nM concentration of thapsigargin was added to rat fibroblasts at time zero, and the increase in cytoplasmic Ca^{+} concentration was monitored by changes in Fluo-4 fluorescence. Control cells received DMSO. Cells were incubated in Ca^{2+}-free medium, which caused a decrease in cytoplasmic Ca^{2+} at later time points. (B) Thapsigargin decreases the intracellular level of pro-α1(I). Western blotting results with cellular proteins from HSCs treated with 200 nM thapsigargin (lanes 1 to 3) or treated with DMSO (lanes 4 and 5) for the indicated time periods are shown. The blot was probed with anti-pro-α1(I) antibody (top panel) and anti-α-tubulin antibody (bottom panel). A 50-μg aliquot of protein was analyzed under reducing conditions. (C) Secretion of pro-α1(I) into cellular medium of HSCs is blocked by thapsigargin. At time zero cell medium was changed and thapsigargin was added to the cells (lanes 1 to 3), or DMSO was added (lanes 4 to 5). At the indicated time points, an aliquot of the medium was collected and analyzed by Western blotting with pro-α1(I)-specific antibody under reducing conditions. Migration of pro-α1(I) is indicated to the left. (D) The fibronectin level was unaffected by thapsigargin treatment. Cells were treated as described for panel B, and the cellular level of fibronectin was determined by Western blotting (upper panel). The same samples were probed for α-tubulin (lower panel), as a control for loading. (E) Calmidazolium Cl decreases pro-α1(I). The experiment was done as described for that in panel B, except calmidazolium Cl was added at 10 μM. One additional time point was taken at 6 h. Western blotting was done with anticollagen antibody [pro-α1(I)].

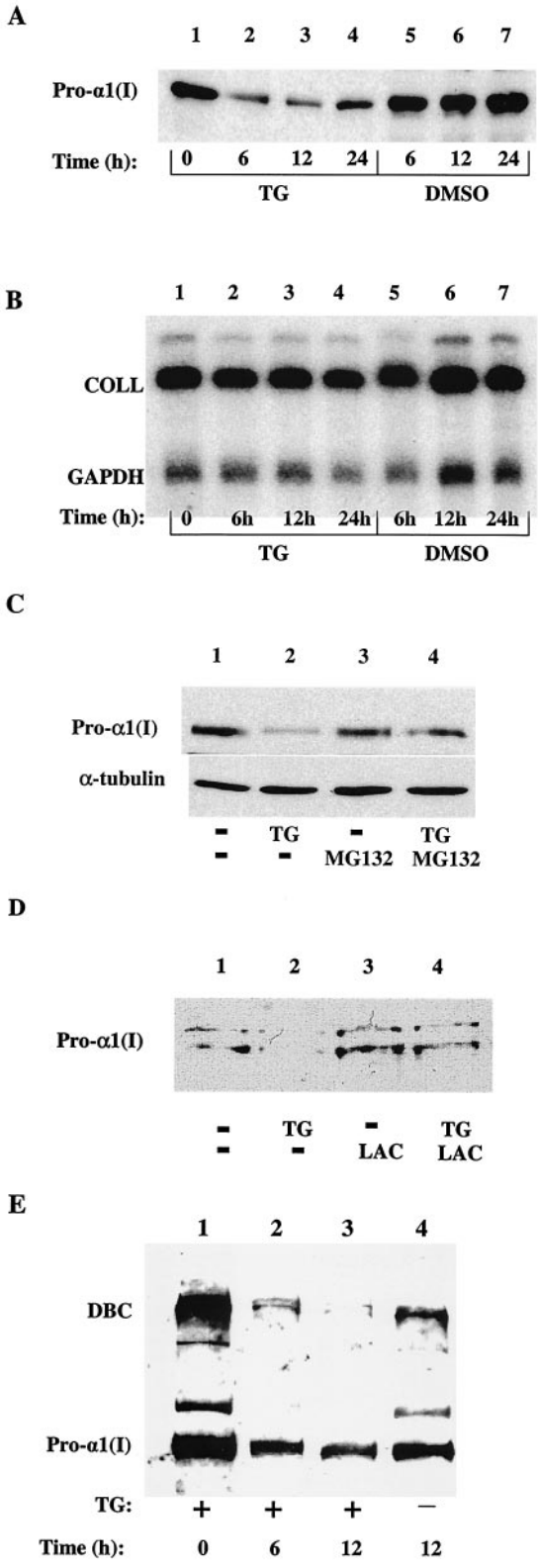


FIG. 7. Inhibition of Serca2b inhibits disulfide bonding and intracellular degradation of pro-α1(I). (A) Thapsigargin causes a transient decrease in the cellular pro-α1(I) steady-state level. Rat fibroblasts were treated with thapsigargin (lanes 1 to 4) or DMSO (lanes 5 to 7) for the indicated time periods. Cellular proteins were analyzed by Western blotting with pro-α1(I)-specific antibody under reducing conditions. (B) Thapsigargin does not affect the steady-state level of collagen α1(I) mRNA. From the same cells as used in panel A, total RNA was extracted and 10 μg was analyzed by RNase protection assay with riboprobe specific to collagen α1(I) mRNA and GAPDH mRNA, as a control. Migration of the specific protected bands is indicated. (C) The proteosome inhibitor MG132 blocked the decrease in intracellular pro-α1(I). Fibroblasts were incubated with DMSO (lane 1), with 200 nM thapsigargin (lane 2), with 100 μM MG132 (lane 3), or with both thapsigargin and MG132 (lane 4) for 6 h. Cellular proteins were extracted and analyzed by Western blotting for pro-α1(I) (top panel) and α-tubulin (bottom panel) under reducing conditions. (D) The proteosome inhibitor lactacystin blocks the thapsigargin-induced decrease in intracellular pro-α1(I). The experiment was done as described for panel C, except that lactacystin was added at 50 μM. Only expression of pro-α1(I) is shown. (E) Thapsigargin completely inhibits formation of disulfide-bonded procollagen. Fibroblasts were incubated with thapsigargin (lanes 1 to 3) for the indicated time periods. Lane 4 represents control cells incubated with DMSO for 12 h. Cellular proteins were extracted, and 50 μg was analyzed by Western blotting under nonreducing conditions with pro-α1(I)-specific antibody. Migration of high-molecular-weight DBC and pro-α1(I) monomers is indicated on the left.

triple helix result in hypermodified chains (4, 49). Thus, synthesis of type I collagen seems to be a tightly regulated process. In this paper we provided insight into a role of the TRAM2 protein in collagen synthesis. We have shown that (i) TRAM2 is a homolog of TRAM, which is an integral part of the translocon (28), but it has a unique C terminus; (ii) TRAM2 is dramatically upregulated during activation of HSCs, a process associated with increased synthesis of type I collagen; (iii) the unique C-terminal region of TRAM2 interacts with the Ca^{2+} pump of the ER, Serca2b, and deletion of this region converts TRAM2 into an inhibitor of procollagen type I synthesis; (iv) maintaining a high Ca^{2+} concentration in the ER is critical for folding and secretion of type I procollagen; and (v) unfolded type I collagen is degraded intracellularly by a proteosome-mediated mechanism.

When quiescent HSCs are compared to HSCs cultured for 7 days, there is a 50- to 60-fold increase in the steady-state level of COLL1A1 mRNA (46). Type I procollagen protein cannot be detected by Western blotting in quiescent HSCs, and its accumulation starts at day 4 in culture with a steady increase until day 7 (unpublished results). To sustain such an increase in procollagen synthesis, HSCs must upregulate components of the collagen biosynthetic pathway. Molecular chaperones are necessary for folding of procollagen type I, and it has been documented that an increase in HSP47, a collagen-specific molecular chaperone, parallels the increase in collagen synthesis in various tissues (14, 38, 40, 55). The number of translocons per cell must also increase during activation of HSCs, because rough ER becomes more prominent during their activation into myofibroblast-like cells (17, 50). In our microarray study we detected upregulation of the Sec 10-like 1 gene and Sec 23 homolog B gene, encoding components of the protein secretion pathway. Three Ca^{2+} binding proteins were also upregulated on our microarray: calmodulin, reticulocalbin, and calpain. TRAM is an integral part of translocon, but the function of this protein is not clear. In vitro reconstitution experiments have shown that it is required for translocation of some proteins, but not all, across the lipid bilayer (21). One report suggested that it controls the exposure of nascent ApoB

polypeptide to the cytoplasm during the translocation process (24). TRAM2 has a high degree of homology to TRAM, except for the C-terminal domain. TRAM2 is upregulated during activation of HSCs (Fig. 2), and this parallels the increase in procollagen synthesis, while TRAM mRNA is not changed in activation of HSCs. We found that TRAM2 interacts with pro-α1(I) chains, suggesting that it may control their translocation. The unique amino acid sequence of the C-terminal domain of TRAM2 (Fig. 1) suggests a specific function. We have described here that this domain interacts with the main Ca^{2+} pump of the ER, Serca2b (10, 27) (Fig. 3). We favor the hypothesis that, besides the direct interaction of TRAM2 with pro-α1(I), the interaction between TRAM2 and Serca2b couples activity of the translocon to the local Ca^{2+} concentration in the ER. When we overexpressed a truncated version of TRAM2, it inhibited procollagen type I synthesis and secretion by HSCs (Fig. 4 and 5). One explanation for this may be that the truncated TRAM2 displaced the endogenous TRAM2 from the translocons, thus preventing their interaction with Serca2b. This may lead to disturbed Ca^{2+} homeostasis in the vicinity of the translocons and inappropriate function of molecular chaperones. Molecular chaperones require Ca^{2+} for function, and some are Ca^{2+} binding proteins (2, 15, 34, 35). Impaired function of molecular chaperones may lead to inefficient folding of the procollagen triple helix, resulting in a block to secretion and increased intracellular degradation. Consequently, the change in HSC morphology upon overexpression of the truncated form of TRAM2 may be due to the triggered UPR (22, 41). Even if the UPR had been triggered, the activation of HSCs was not affected, since expression of α-SMA, as another marker of HSC activation, was unchanged, suggesting that the cells were differentiating. An alternative explanation for the inhibitory effect of TRAM2ΔC may be that the protein disturbs the assembly or geometry of translocons, rendering them nonfunctional without affecting the function of molecular chaperones. However, experiments with thapsigargin and calmidazolium Cl suggest that the high Ca^{2+} concentration within the ER is necessary for triple helical procollagen assembly. Inhibition of triple helical procollagen assembly, as assessed by high-molecular-weight DBC, is completely abolished by thapsigargin and temporally precedes a decrease in the steady-state level of pro-α1(I) monomers (Fig. 7). Also, secretion of procollagen into cellular medium ceases almost immediately after addition of thapsigargin (Fig. 6). This suggests that assembly of procollagen triple helices is Ca^{2+} dependent and is consistent with the hypothesis that one of the functions of TRAM2 may be to recruit Serca2b to the translocon, which leads to increased local Ca^{2+} concentration and stimulation of chaperone-mediated folding of triple helical procollagen. Thus, one of the physiological roles of TRAM2 would be to couple activity of translocon to activation of molecular chaperones.

TRAM2 is expressed in most cell types (39), where it may have similar function in folding of proteins other than collagen. In HSCs and fibroblasts, which secrete primarily collagen type I, its role may be predominantly in procollagen synthesis, as suggested by coprecipitation of TRAM2 and pro-α1(I). Existence of yet another homolog of TRAM, the "protein similar to TRAM" (accession number BC 030831), suggests that there is a family of TRAM-related proteins with distinct functions in protein translocation. However, coupling of Serca2b to the translocon may be the specific function of TRAM2.

The decrease in intracellular pro-α1(I) induced by thapsigargin was inhibited by the proteosome inhibitors MG132 and lactacystin (Fig. 7). This suggests that Ca^{2+} depletion leads to increased intracellular procollagen degradation. Studies of fibroblasts derived from osteogenesis imperfecta patients have shown that unassembled procollagen polypeptides undergo intracellular degradation by a proteosome-dependent mechanism (18). Our experiments with two different proteosome inhibitors confirmed that the cytoplasmic proteosome is involved in degradation of unassembled pro-α chains. This implies that unassembled pro-α chains must be retrotranslocated into the cytoplasm. How procollagen chains are targeted for destruction and returned to the cytoplasm is unknown. One possibility is that the polypeptides remain associated with the ER membrane until proper modifications are made and until they initiate productive folding into the triple helix. In such a case, misfolded or hypermodified chains would be reloaded into the translocon and reverse translocated into the cytoplasm for degradation. Elucidation of this mechanism has profound implications on how the biosynthesis of type I procollagen is regulated and if it takes place entirely on the membrane of the ER. Folding of type III procollagen could be achieved when its C-terminal domain was replaced with a single transmembrane domain of HA (8). Thus, procollagen folding can take place on the membrane of the ER. If procollagen synthesis is membrane bound, this would greatly increase the local concentration of the components involved. It has been reported that lysyl hydroxylase is associated with the membrane of the ER (48), and our investigators have postulated that COLL1A1 mRNA and COLL1A2 mRNA may be targeted for coordinated translation by the conserved 5′ stem-loop (44). Elucidation of all components of the translocation-synthesis machinery for type I collagen is, therefore, an important future goal.

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