

Sec61alpha synthesis is enhanced during translocation of nascent chains of collagen type IV in F9 teratocarcinoma cells after retinoic acid treatment

L.R. Ferreira¹,
C.E.E. Velano²,
E.C. Braga²,
C.C. Paula²,
H. Martélli Junior³
and J.J. Sauk⁴

¹Grupo de Pesquisa em Biomedicina, Departamento de Ciências Morfológicas, Pontifícia Universidade Católica de Minas Gerais, Poços de Caldas, MG, Brasil
²Departamento de Clínica Médica, Faculdade de Medicina, Universidade de Alfenas, Alfenas, MG, Brasil
³Departamento de Periodontia, Faculdade de Odontologia, Universidade de Alfenas, Varginha, MG, Brasil and Universidade de Montes Claros, Montes Claros, MG, Brasil
⁴Department of Oral Pathology, Dental School, University of Maryland, Baltimore, MD, USA

Abstract

Correspondence

L.R. Ferreira
Rua Manoel Pedro Rodrigues, 145
37130-000 Alfenas, MG
Brasil
E-mail: resende@pucpcaldas.br

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Nascent procollagen peptides and other secretory proteins are transported across the endoplasmic reticulum (ER) membrane through a protein-conducting channel called translocon. Sec61 α , a multispanning membrane translocon protein, has been implicated as being essential for translocation of polypeptide chains into the cisterns of the ER. Sec61 α forms a protein complex with collagen and Hsp47, an ER-resident heat shock protein that binds specifically to collagen. However, it is not known whether Sec61 α is ubiquitously produced in collagen-producing F9 teratocarcinoma cells or under heat shock treatment. Furthermore, the production and utilization of Sec61 α may depend on the stage of cell differentiation. Cultured F9 teratocarcinoma cells are capable of differentiation in response to low concentrations of retinoic acid. This differentiation results in loss of tumorigenicity. Mouse F9 cells were grown in culture medium at 37°C and 43°C (heat shock treatment) treated or not with retinoic acid, and labeled in certain instances with ³⁵S-methionine. Membrane-bound polysomes of procollagen IV were then isolated. Immunoprecipitation and Western blot analysis were performed using polyclonal antibodies against collagen IV, Hsp47 and Sec61 α . Under retinoic acid-untreated conditions, F9 cells produced undetectable amounts of Sec61 α . Sec61 α , Hsp47 and type IV collagen levels were increased after retinoic acid treatment. Heat shock treatment did not alter Sec61 α levels, suggesting that Sec61 α production is probably not affected by heat shock. These data indicate that the enhanced production of Sec61 α in retinoic acid-induced F9 teratocarcinoma cells parallels the increased synthesis of Hsp47 and collagen type IV.

Key words

- Sec61 α
- Collagen type IV
- Retinoic acid
- F9 cells
- Differentiation

Introduction

Secretory proteins are transported across the endoplasmic reticulum (ER) membrane through a protein-conducting channel (1,2). This protein-conducting channel consists of ER-associated proteins forming a translocon, which includes the translocon-associated protein, the translocating chain-associating membrane protein (TRAM) and the Sec61p complex (1,2). TRAM and Sec61p are tightly associated with membrane-bound ribosomes, suggesting that polypeptide nascent chains pass directly from the ribosome into a protein-conducting channel (3,4).

The Sec61p complex is composed of three subunits: α , β , γ (5). Sec61 α , a multispanning membrane protein and the largest component of the Sec61p complex, is essential for translocation of polypeptide chains through this channel into the cisterns of the ER (5). The Sec61p complex is homologous to SecYp present in bacteria (6).

The translocation process requiring the Sec61p complex in mammalian cells is associated with a specialized co-translational pathway in which the nascent polypeptide is continuously bound to polysome-bound ribosomes as it translocates through the ER membrane (7). Photocrosslinking experiments and studies involving chemical cross-linking reagents such as dithio-bis-succinimidyl propionate (DSP) or disuccinimidylsuberate have shown that Sec61 α is closely associated with nascent polypeptides undergoing translocation across mammalian rough ER including collagen and Hsp47 (3,8).

Hsp47 is a 47-kDa collagen-binding glycoprotein localized in the ER of collagen-producing cells (9). Hsp47 binds primarily to collagens, procollagen in particular, but may also associate with fetuin (10). Hsp47 is constitutively expressed, as well as affected by environmental stresses such as heat shock (11,12). Following heat shock, Hsp47 protein levels rapidly increase through an acti-

vation of heat shock transcription factors (13). Immunoprecipitation studies have revealed that Hsp47 is closely associated with the translation-translocation machinery during the production of procollagen I, suggesting that Hsp47 might function as a molecular chaperone for nascent chains of collagen (14,15). The levels of Hsp47 have been shown to vary with collagen concentration following retinoic acid-induced differentiation of mouse teratocarcinoma cells (16), in pathological conditions such as hepatic, renal and pulmonary fibrosis (17-20), and in cultured fibroblasts grown from patients with hereditary gingival fibromatosis (21).

In the present study, we tested the proposition that the translocation of secretory proteins through the ER membrane is the result of the enhanced production and utilization of Sec61 α and may depend on the stage of cell differentiation. However, it is not known whether Sec61 α is produced ubiquitously in all collagen-producing cells or under heat shock treatment. Stem cells from many cultured teratocarcinoma cell lines such as mouse F9 and P19 cells are capable of differentiation in response to low concentrations of retinoic acid, a derivative of vitamin A (22). This differentiation of tumorigenic stem cells results in the loss of tumorigenicity (23). Therefore, teratocarcinoma cell lines provide a model to examine the association between the process of cell differentiation and the production of Sec61 α .

We determined the synthesis of Sec61 α and Hsp47 during translocation of collagen nascent chains in F9 cells following retinoic acid and heat shock treatment.

Material and Methods

Cell culture and metabolic labeling

F9 teratocarcinoma cells, obtained from the American Type Tissue Culture Collection (Rockville, MD, USA), were grown to confluence in 75-cm² plastic flasks using

Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum, 1.16 g/l glutamine, and 100 mg/ml streptomycin at 37°C in 5% CO₂. For the cell growth experiments, F9 stem cells (1 x 10⁵ cells) were plated onto gelatinized tissue culture plates containing 10 ml of medium. Mouse F9 cells were induced to differentiate into parietal endoderm cells after treatment with all-trans retinoic acid (10 μ M) for 4 days. When it was necessary to label proteins, the medium was removed and replaced with fresh methionine-free DMEM containing ³⁵S-methionine (100 μ Ci/ml; New England Nuclear, Boston, MA, USA) for 20 min under normal conditions (37°C) or for 90 min at 43°C for heat shock. ³⁵S-methionine was used to label nascent chains of collagen and Hsp47.

Immunoprecipitation and analysis by gel electrophoresis

The ³⁵S-methionine-labeled F9 cells were collected and incubated in lysis buffer (50 mM Tris-HCl, pH 8.0, 40 mM NaCl, 5 mM EDTA) at 4°C for 30 min. Rabbit antibodies against mouse type IV collagen, Hsp47, and Sec61 α (diluted 1:2000) were added to the samples. The samples (100 μ l) were incubated for 2 h at 4°C, then precipitated with 30 μ l of protein A-Sepharose. The precipitated immune complexes were washed three times with 1 ml of lysis buffer, and once with 1 ml of 10 mM Tris-HCl, pH 6.8. The samples were suspended in Laemmli's sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (1.25 M Tris-HCl, pH 6.8, 10% SDS in distilled water, 30% bromophenol blue in glycerol and 0.5% mercaptoethanol), and boiled for 5 min. Twenty-five microliters of the supernatant was loaded onto 4-20% polyacrylamide gels by the method of Laemmli (24). The gels were fixed, dried and autoradiographed using the method of Bonner and Laskey (25). In some instances, treatment with a cleavable bifunctional membrane permeable cross-linking

reagent, DSP, was performed prior to immunoprecipitation (16). After labeling, cells were harvested and rinsed with phosphate-buffered solution (PBS). Cells were then treated with 0.1% collagenase to remove the extracellular collagen. After rinsing in PBS, cells were combined with 2 ml DSP (0.1% in DMSO) and placed on ice for 30 min. After 30 min the cells were rinsed with 2 mM glycine in PBS and lysed in lysis buffer with 50 μ g/ml gelatin on ice for 15 min. The supernatants were then divided into equal aliquots and immunoprecipitation was performed as described above.

Isolation of membrane-bound polysomes (microsomes) and collagenase digestion

Microsomes from F9 cells were isolated by the method of Vedeler et al. (26). After microsomes were collected, immunoprecipitation was performed as previously described. In some cases, collagenase digestion was performed before adding Laemmli's buffer. Microsome extracts were incubated with 2.7 U of bacterial collagenase in 50 μ l collagenase buffer (10 mM Tris, pH 7.5, 40 mM NaCl, 5 mM CaCl₂, 30 mM KCl, 10 mM PMSF, 50 μ M N-ethylmaleimide, 2.5 mM benzamidine) and incubated for 1 h at 37°C. Laemmli's buffer was then added to the samples, the samples were boiled for 5 min and the immunoprecipitated microsomes bound to protein A-Sepharose were released and collected. SDS-PAGE and autoradiography were performed as previously described.

Western blotting by enhanced chemiluminescence

F9 microsomes were isolated and analyzed by SDS-PAGE as described above. The microsomes were then electrotransferred to a nitrocellulose filter (27). The filter was blocked for 1 h with 10% dried milk in PBS and rinsed three times with PBS. Anti-

bodies against TRAM or Sec61 α (Biopolymer Laboratories, Baltimore, MD, USA) diluted 1:2,000 in 5% dried milk/1% BSA in PBS were added, and the filter was incubated overnight at room temperature. The filter was washed four times with PBS in 0.2% Tween 20 and incubated for 2 h at room temperature with a secondary goat anti-mouse IgG antibody (Amersham Inc., Arlington Heights, IL, USA) diluted 1:2000 in 1% BSA in PBS, in order to reduce non-specific binding according to Towbin et al. (27). The filter was then washed in PBS and incubated with enhanced chemiluminescence reagents for 1 min according to the manufacturer protocol (Amersham). An X-ray film was exposed to the filter for less than a minute.

Results

It is not known whether Sec61 α is ubiquitously expressed and produced in collagen-producing cells. Furthermore, it is also not clear whether this binding occurs through the enhanced production and utilization of Sec61 α and whether it might be dependent on the stage of cell differentiation. In order to answer these questions, we treated F9 cells with all-trans retinoic acid for 4 days, followed by labeling with ^{35}S -methionine. Membrane-bound polysomes (microsomes) were extracted from the cells according to the method of Vedeler et al. (26). The effect of all-trans retinoic acid on the synthesis of Hsp47 (47 kDa), collagen IV (170- to 185-kDa bands which correspond to $\alpha 2$ and $\alpha 1$ chains, respectively) and Sec61 α is shown in Figure 1. Anti-Sec61 α antibodies immunoprecipitated a protein of approximately 37-38 kDa in samples treated with retinoic acid (Figure 1, lane 4), while no bands were detected in control samples (Figure 1, lane 3). Treatment of F9 cells with retinoic acid resulted in increased collagen and Hsp47 synthesis (Figure 1, lane 6).

To assess whether Sec61 α is closely associated with Hsp47 and nascent collagen chains, a cross-linking agent, DSP, was added to the samples prior to immunoprecipitation (Figure 2). Anti-Hsp47 antibodies immunoprecipitated Hsp47 (47 kDa) and collagen IV (170-185 kDa) (Figure 2, lane 1). The 94-kDa band also shown in Figure 2 (lane 1) is probably Grp94. Grp94 (glucose-related protein, 94 kDa) is an ER-resident protein known to form protein complexes with collagen and Hsp47 in previous studies (14,16). Anti-Sec61 α antibodies immunoprecipitated Sec61 α and also Hsp47, collagen IV, and Grp94 (Figure 2, lane 2). Treatment of the samples with collagenase digested the high molecular weight bands (170 and 185 kDa), confirming that these, in fact, correspond to collagen chains (Figure 2, lanes 3 and 4).

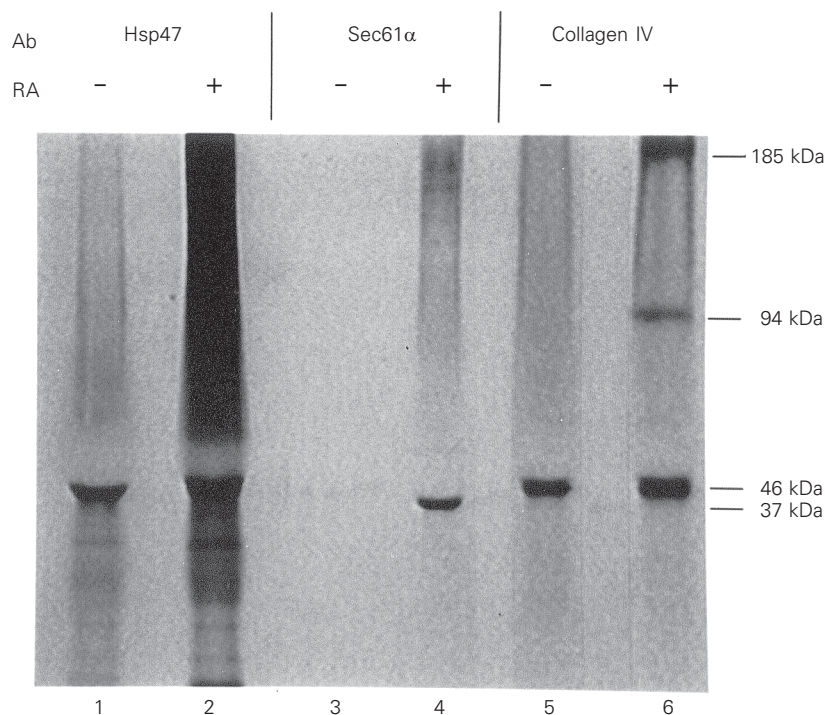


Figure 1. Immunoprecipitation of membrane-bound polysomes (microsomes) with anti-Hsp47, anti-Sec61 α , and anti-collagen IV antibodies after retinoic acid treatment of F9 cells. Cells treated (lanes 2, 4 and 6) or not (lanes 1, 3 and 5) with retinoic acid for 4 days were labeled for 1 h with ^{35}S -methionine. Microsomes were immunoprecipitated with anti-Hsp47 (lanes 1 and 2), anti-Sec61 α (lanes 3 and 4), and anti-collagen IV (lanes 5 and 6) antibodies. Ab: antibody; RA: all-trans retinoic acid. Molecular masses of standard proteins are given on the right side of the figure.

To confirm the presence of both TRAM and Sec61 α in F9 cell membrane-bound polyosomes after treatment with retinoic acid, enhanced chemiluminescence Western blotting using specific antibodies against TRAM (28) and Sec61 α was employed (Figure 3). Sec61 α was detected in retinoic acid-treated (Figure 3, lane 4) but not in untreated cells (Figure 3, lane 3). Interestingly, the other component of the protein-conducting channel, TRAM, was seen in both control and retinoic acid-treated samples (Figure 3, lanes 1 and 2). TRAM was previously shown to mediate protein translocation in mammalian cells (28).

It was also important to determine whether Sec61 α production is influenced by heat shock. Hsp47 is constitutively expressed, and also has its production increased by stress including heat shock (29). After heat shock, Hsp47 protein levels rapidly increase through an activation of heat shock transcriptional factors (13). Western blots using anti-Sec61 α antibodies demonstrated Sec61 α in samples from both control and heat shock-treated cells following retinoic acid treatment (Figure 4, lanes 2 and 4). Sec61 α was not detected in untreated control cells (incubated at 37°C; Figure 4, lane 1) or in retinoic acid nontreated heat-shocked cells (incubated at 43°C; Figure 4, lane 3).

Discussion

Retinoic acid is an established regulator of epithelial cell differentiation *in vivo* and *in vitro* (30). One of the major physiological functions of retinoic acid is to prevent squamous differentiation (23,31). Squamous metaplasia, which develops during vitamin A deficiency, can be reversed by supplementation with retinoic acid (23). Furthermore, retinoic acid suppresses squamous cell differentiation that occurs spontaneously or induced by calcium ions in cultured normal keratinocytes or tracheal cells (23).

F9 cells comprise a cultured teratocarci-

noma cell line which can be induced to differentiate into parietal endodermal cells (primitive endoderm-like cells) in the presence of retinoic acid and dibutyryl cAMP (32). The phenotype of the new cell type is characterized by synthesis of plasminogen activator, laminin and type IV collagen and by low levels of alkaline phosphatase and lactase dehydrogenase. The cell type generated by this induction is not dependent upon

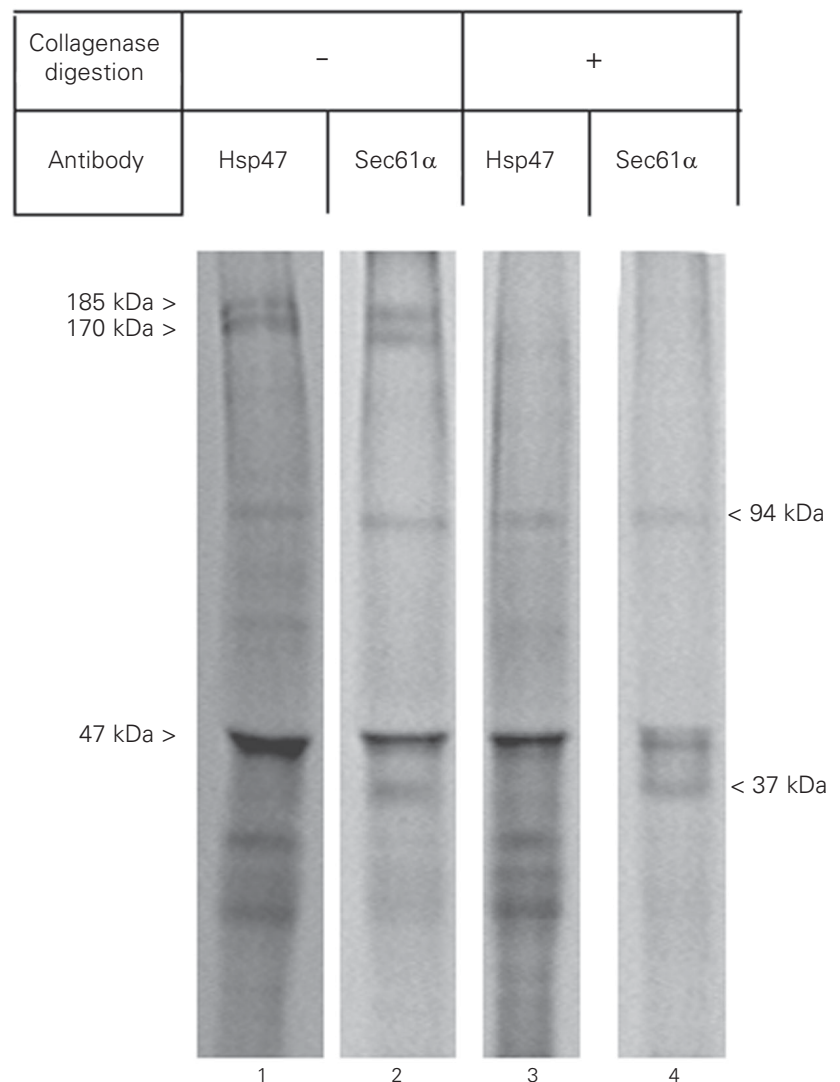


Figure 2. Immunoprecipitation with anti-Hsp47 and anti-Sec61 α antibodies and collagenase digestion of retinoic acid-treated membrane-bound collagen IV microsomes following treatment with DSP. Microsomes were immunoprecipitated with anti-Hsp47 (lanes 1 and 3) and anti-Sec61 α (lanes 2 and 4) antibodies. Collagenase digestion completely disrupted collagen microsomes (lanes 3 and 4).

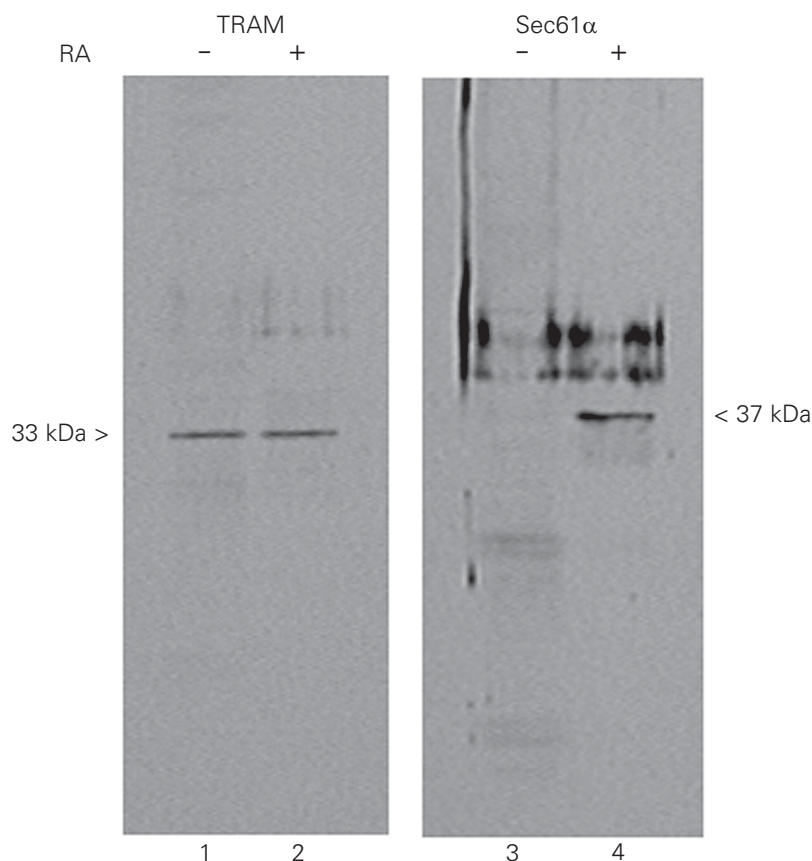


Figure 3 - Distribution of translocating chain-associating membrane protein (TRAM) and Sec61 α using the Western blotting technique with enhanced chemiluminescence following retinoic acid (RA) treatment. F9 cells were incubated at 37°C with (lanes 2 and 4) or without (lanes 1 and 3) retinoic acid for 4 days. Antibodies against TRAM (lanes 1 and 2) and Sec61 α (lanes 3 and 4) were applied and the nitrocellulose paper was then exposed to radiographic film.

the continued presence of either compound, but the cAMP agents are active only on F9 cells that have been treated with retinoic acid (33,34). This differentiation of the tumorigenic F9 stem cells results in the loss of their tumorigenicity (32). This differentiation response of F9 cells is irreversible, fairly synchronous, and rapid within 24 to 48 h (34).

It is unclear at the present time whether an intracellular binding protein mediates all of the actions of retinoic acid in cells. There has also been no convincing evidence in any system that retinoic acid regulates transcription of specific genes including Sec61 α . Although the mechanism whereby retinoic acid regulates gene expression and differentiation is not completely understood, it is likely that high-affinity retinoic acid receptors and/or binding proteins may be involved.

The delineation of translocation mechanisms in mammalian cells is still incomplete. However, several investigators have provided evidence that the ER membrane glycoproteins TRAM and the Sec61p complex are components of the mammalian ER translocon that participates in protein translocation (2). These translocon proteins may associate with ER proteins during the translocation of secretory proteins. Candidates for this asso-

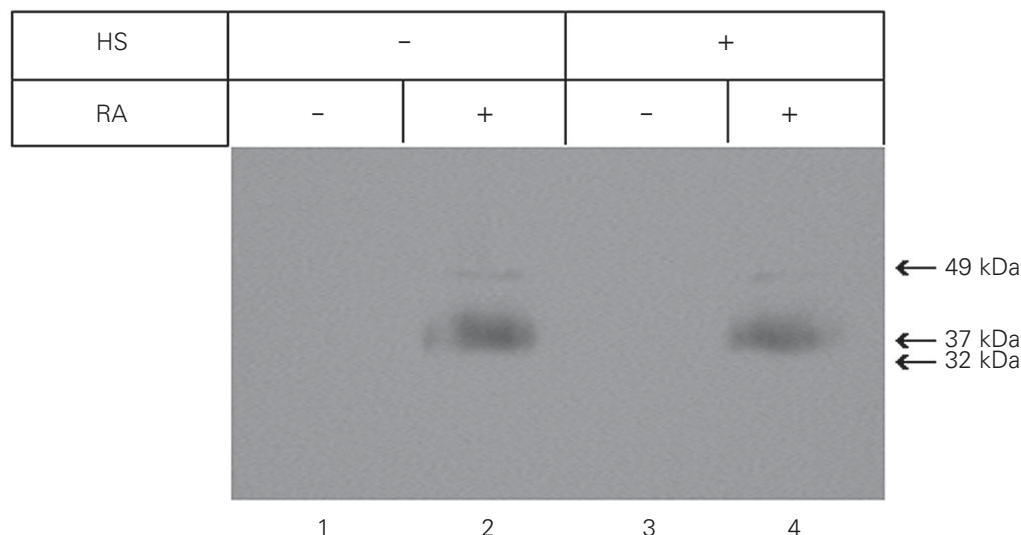


Figure 4. Distribution of Sec61 α using the Western blotting technique with enhanced chemiluminescence following retinoic acid (RA) and heat shock (HS) treatment. F9 cells were either incubated at 37°C (control cells, lanes 1 and 2) or at 43°C (heat-shocked cells, lanes 3 and 4) for 90 min. In some cases, F9 cells were treated with all-trans retinoic acid for 4 days (lanes 2 and 4). Antibodies against Sec61 α were added and the nitrocellulose paper was then exposed to radiographic film.

ciation include components of the ribosome-binding site or the major component of the mammalian homolog of the Sec61p complex, Sec61 α (2). The precise localization of Sec61 α in the ER membrane has not been determined, although it is likely that portions of Sec61 α project into the ER lumen. Sec61 α was localized adjacent to nascent polypeptides that are being translocated through the ER membrane (8). Since Sec61 α proteins were not associated with long translating polypeptide chains, it has been suggested that Sec61 α proteins reside near nascent chains at the beginning of their membrane passage (2). Furthermore, Sec61 α was essential for the translocation of certain nascent polypeptide chains through the ER membrane (2).

It is not clear whether Sec61 α binding to translocating proteins occurs through the enhanced production and utilization of Sec61 α , or whether it is dependent on the stage of cell differentiation. It is also not known to date whether Sec61 α is constitutively expressed and produced in collagen-producing cells. Accordingly, in the present study we investigated the production of Sec61 α and other translocon components following retinoic acid treatment. The production of Sec61 α markedly increased after retinoic acid treatment. Sec61 α production was not detected in untreated F9 cells. However, another translocon component, TRAM, was present in both retinoic acid-treated and control samples.

The relevance of the undetectable levels of Sec61 α in undifferentiated F9 cells is not known. Sec61 α was essential for the translocation of secretory proteins (2). The mechanism whereby these tumor cells secrete proteins without detectable amounts of Sec61 α is still not well understood. Further experiments are needed to clarify the role of Sec61 α in collagen-producing cells. Among mammalian species, the cDNA sequence for the Sec61 α gene is known for rats, dogs, and humans, but the cDNA sequence for the

mouse Sec61 α gene has not been reported.

A number of explanations currently exist for the source of the forces that drive the newly synthesized chains into the lumen of the ER. Topogenic sequences located in translocon components may influence translocation into the ER (35,36). However, another possibility is that simple elongation of the nascent chain drives extrusion of the polypeptide into the ER and the protein translocation channel is a passive pore (37). Consequently, this channel is without directional preference for polypeptide movement. Thus, the bias in random movement of a polypeptide resulting in vectorial translocation is driven by nascent chain elongation and is sustained by inclusion of a stop codon or by post-translational events, such as glycosylation, folding and/or binding to chaperones (38). While it is not known whether distinctive chaperones or translocons are utilized for specialized proteins such as procollagen, there is strong evidence to suggest that some classes of proteins use distinctive machinery for their organization. For example, although evidence strongly indicated the participation of TRAM in the translocation of β -lactamase and prepro- α -factor, elimination of this translocon component only reduced the translocation efficiency for preprolactin (3).

Using a cross-linking reagent on membrane-bound polysome extracts from retinoic acid-induced F9 teratocarcinoma cells, Sec61 α is shown in this study to co-precipitate with collagen IV, Hsp47, and Grp94. These results indicate that a major component of the translocon complex, Sec61 α , may associate early with nascent collagen IV polypeptides and ER-resident molecular chaperones during translation/translocation. These data suggest that Sec61 α may form complexes with Hsp47 and other ER proteins during the constitutive synthesis of collagen IV by F9 cells after retinoic acid treatment.

Hsp47, a collagen-bound heat shock protein, has been found associated with evolu-

ing chains of procollagen associated with polysomes by binding to the Gly-X-Y regions of nascent collagen chains (39). Sauk et al. (15) have demonstrated that Hsp47 associates with Grp78, Grp94 and nascent procollagen I to form large complexes. Gorlich et al. (40) have shown the association of TRAM and Sec61 α with complexes of proteins after photocrosslinking. High et al. (3) demonstrated the formation of protein complexes with Sec61 α after chemical cross-linking with DSP. These data support the hypothesis of a successive action of molecular chaperones.

The production of Sec61 α was shown to

be markedly increased following retinoic acid treatment. Another translocon component, TRAM, was demonstrated to be present in both retinoic acid-treated and control samples. Therefore, the absence or limited production of Sec61 α in F9 cells prior to retinoic acid treatment is not restricted to other members of the translocation machinery. The relevance of the diminished or absent production of Sec61 α in undifferentiated F9 cells is not known. Also, the mechanism whereby these tumor cells secrete proteins without detectable amounts of Sec61 α is still not well understood.

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