Interaction of tissue transglutaminase with nuclear transport protein importin-α3

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Abstract Tissue transglutaminase is a multifunctional enzyme which has been involved in the regulation of cell growth, differentiation, and apoptosis. Recently, nuclear localization of tTG has been reported indicating the potential of active nuclear transport. In this study we use the yeast two-hybrid assay and co-immunoprecipitation to show that tTG interacts with the nuclear transport protein importin-α3. Using electron microscopy we demonstrate that nuclear expression of tTG in a non-small cell lung cancer cell line is induced by retinoic acid (RA). These data suggest that importin-α3 could mediate active nuclear transport of tTG which may be important for the regulation of critical cellular processes.

Key words: Tissue transglutaminase; Importin; Nuclear transport

1. Introduction

Tissue transglutaminase (tTG), or type II transglutaminase, is an 80 kDa enzyme that has been implicated in the regulation of cell growth [1,2], differentiation [3,4], and apoptosis [5,6]. In keeping with multiple physiological roles for tTG are the numerous findings that expound its importance in tumor growth and progression [7,8] and in the pathology of several neurodegenerative diseases characterized with the expansion of CAG (glutamine) repeats, such as Huntington's disease [9]. tTG is a member of a family of Ca^{2+}-dependent enzymes that catalyze protein cross-linking reactions by creating ε-(γ-glutamyl)lysine bonds between the γ-carboxyl group of a glutamine residue in one polypeptide chain and the ε-amino group of a lysine residue in a second polypeptide chain [10]. tTG possesses an additional feature that makes it unique among other transglutaminases, namely, the ability to catalyze GTP hydrolysis enabling it to participate in signal transduction by functioning as an α-subunit of a G_h-protein [11,12]. The G-protein activity of tTG has been shown to be critical for its role in cell cycle progression [13]. It is yet unknown which function of tTG, transglutaminase, G-protein, or both, is responsible for its roles in cell growth, apoptosis and differentiation, nor are the mechanisms regulating these functions completely understood.

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Abbreviations: tTG, tissue transglutaminase; NSCLC, non-small cell lung cancer; RA, all-trans retinoic acid; EM, electron microscopy

tTG has been localized mainly in the cytosol, however, a detectable tTG expression, both as a cross-linking enzyme and a G-protein, has been reported in the nucleus [14,15]. Two nuclear substrates of the transglutaminase activity of tTG, histones [16] and retinoblastoma protein (Rb) [17], have been identified so far. Modification of both substrates by polyamination or cross-linking may be relevant to the role of tTG in cell cycle progression and apoptosis. Direct targets of the G-protein activity of nuclear tTG are unknown. The mechanisms of nuclear translocation of tTG have not been yet demonstrated. We show here that the nuclear transport protein, importin-α3, is a specific cellular binding partner for tTG, thus establishing a molecular mechanism of active nuclear transport of tTG.

2. Materials and methods

2.1. Yeast two-hybrid assay

The Matchmaker II two-hybrid system (Clontech) was used to screen a human liver cDNA library cloned into GAL4 activation domain (AD) of pACT2 plasmid (Clontech). The bait construct was created by subcloning the full length tTG gene into the GAL4 DNA-binding domain (DNA-BD) of pAS2-1 plasmid by ligating the *Hin*dIII-*Eco*RI fragment from pIND (Invitrogen)-tTG cDNA into the *Nde*I-*Eco*RI digested pAS2-1. The screening was performed according to the manufacturer's protocol. Sequencing of the isolated cDNA was performed using the ABI Prizm Automated Sequencer (Model 377). We utilized two primers, AGTTGAAGTGAACTTGCGG and CAGATTACGCTAGCTTGGG. The BLAST search algorithm was employed for sequence comparisons.

2.2. In vitro translation and protein-binding assay

Importin-α3 partial cDNA was excised from the pACT2 plasmid and subcloned downstream from the T7 RNA polymerase promoter in a PCR3-Uni plasmid (Invitrogen) by ligating the *Sma*I-*Xho*I fragment from $PACT_2$-importin-α3 cDNA. ^{35}S-labeled importin-α3 was synthesized in the TNT coupled reticulocyte transcription/translation system (Promega) according to the manufacturer's protocol.

In vitro translated ^{35}S-labeled importin-α3 was incubated with or without 3.5 μg of purified guinea pig liver tTG (Sigma) in binding buffer (20 mM HEPES pH 7.9, 20% glycerol, 0.5 mM EDTA, 60 mM KCl, 6 mM $MgCl_2$, 0.1% NP-40) for 60 min at 30°C. Mixtures were immunoprecipitated with polyclonal goat anti-guinea pig tTG antibody (Upstate Biotechnology), fractionated on SDS-PAGE, and ^{35}S-labeled band was detected by autoradiography.

2.3. Cell culture

The non-small cell lung cancer (NSCLC) cell line, NCI-H596, was grown in RPMI-1640 media (GIBCO BRL) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL) at 37°C and 5% CO_2. Where indicated, 10^{-5} M all-trans retinoic acid (RA; Sigma Chemical Co.) was added in serum-free medium. RA was initially dissolved in DMSO to a concentration of 10^{-1} M and subsequently diluted in RPMI medium without FBS to a final concentration of 10^{-5} M.

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2.4. Immunoprecipitation and Western blot analysis

NCI-H596 cells were lysed by the addition of RIPA immunoprecipitation buffer (10 mM HEPES pH 7.5, 50 mM KCl, 2.5 mM $MgCl_2$, 1% Triton X-100, 1.5 mM EDTA, 0.1 mM Na_3V0_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). tTG was immunoprecipitated for 1 h at 4°C using polyclonal goat anti-guinea pig tTG antibody (Upstate Biotechnology) or monoclonal antibody Cub 7401, specific for tTG (gift from Dr. P. Birckbichler, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA) immobilized on Protein A/G agarose beads (Calbiochem). Importin-α3 was immunoprecipitated with specific polyclonal rabbit antibody (generous gift of Dr. E. Hartmann, Max-Delbrück-Centrum, Berlin, Germany). Immunoprecipitated proteins were subjected to Western blot analysis with anti-importin-α3 for tTG immunoprecipitates and anti-tTG for importin immunoprecipitates. The proteins were visualized using enhanced chemiluminescence (ECL) system (Amersham).

2.5. Electron microscopy

Confluent NCI-H596 cells were treated with or without 10^{-5} M all-trans retinoic acid (RA) for 0, 6 and 24 h. Cells were fixed with glutaraldehyde, embedded in LRWhite (London Resin Co.) and tTG was immunolocalized with monoclonal antibody Cub 7401 or, as a negative control, with monoclonal antibody BC.1 specific for keratinocyte transglutaminase (kTG) (gift from Dr. S. Thacher, Allergan, Irvine, CA, USA) followed by staining with anti-mouse IgG-conjugated immunogold 10 nm particles. Electron microscopy was performed using Philips SM10 electron microscope.

2.6. Immunohistochemistry

NCI-H596 cells were trypsinized and cytospins prepared. Cells were permeabilized with methanol and saponin. tTG was immunolocalized using monoclonal Cub 7401 antibody (1:20) for 1 h on ice, followed by incubation with FITC-conjugated anti-mouse IgG (1:100, Vector) for 2 h. Importin-α3 was immunolocalized using rabbit polyclonal antibody (1:20) for 1 h on ice, followed by incubation with TRITC-conjugated anti-rabbit IgG (1:100; Vector). Images were visualized and superimposed using the Axoplan 2 (Zeiss) confocal fluorescent microscope and QED (QED, Inc.) image analysis system.

3. Results and discussion

3.1. Identification of importin-α3 as a tTG-binding partner using the yeast two-hybrid assay

To fully characterize the role of tTG in cellular processes it is necessary to identify its intracellular targets. So far the tTG targets were identified as the substrates of its transamidase activity [16,17]. To detect other possible targets of tTG we used the yeast two-hybrid assay which has been shown to be effective in detection of weak and transient protein-protein interactions. We performed a two-hybrid screen with human

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    1   *   10    *   20    *   30    *   40    *   50
  1 MAEELVLERCDLELETNGRDHHTADLCREKLVVRRGQPFWLTLHFEGRNY
 51 QASVDSLTFSVVTGPAPSQEAGTKARFPLRDAVEEGDWTATVVDQQDCTL
101 SLQLTTPANAPIGLYRLSLEASTGYQGSSFVLGHFILLFNAWCPADAVYL
151 DSEEERQEYVLTQQGFIYQGSAKFIKNIPWNFGQFQDGILDICLILLDVN
201 PKFLKNAGRDCSRRSSPVYVGRVGSGMVNCNDDQGVLLGRWDNNYGDGVS
251 PMSWIGSVDILRRWKNHGCQRVKYGQCWVFAAVACTVLRCLGIPTRVVTN
301 YNSAHDQNSNLLIEYFRNEFGEIQGDKSEMIWNFHCWVESWMTRPDLQPG
351 YEGWQALDPTPQEKSEGTYCCGPVPVRAIKEGDLSTKYDAPFVFAEVNAD
401 VVDWIQQDDGSVHKSINRSLIVGLKISTKSVGRDEREDITHTYKYPEGSS
451 EEREAFTRANHLNKLAEKEETGMAMRIRVGQSMNMGSDFDVFAHITNNTA
501 EEYVCRLLLCARTVSYNGILGPECGTKYLLNLTLEPFSEKSVPLCILYEK
551 YRDCLTESNLIKVRALLVEPVINSYLLAERDLYLENPEIKIRILGEPKQK
601 RKLVAEVSLQNPLPVALEGCTFTVEGAGLTEEQKTVEIPDPVEAGEEVKV
651 RMDLVPLHMGLHKLVVNFESDKLKAVKGFRNVIIGPA
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Fig. 1. Identification of putative NLS-like sequences in tTG. NLS-like amino acid sequences were identified in tTG using the PROWL search algorithm. Residues 597–602 (bold) are 100% similar to the 203–237 PKQKRK NLS sequence of influenza virus NS1 protein. Residues 259–263 (bold) are 80% similar to the 34–38 DRLRR NS1 sequence.

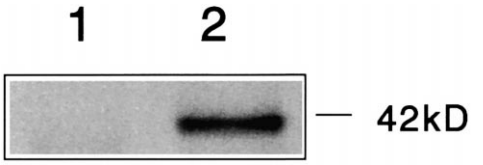

Fig. 2. Binding of in vitro translated [^{35}S]importin-α3 to purified tTG. Position of MW standard (in kDa) is at right. In vitro translated [^{35}S]methionine-labeled importin-α3 (polypeptide derived from the two-hybrid construct) was mixed with purified guinea pig liver tTG, which was then immunoprecipitated with polyclonal goat anti-tTG antibody (lane 2). Non-specific binding of importin-α3 to irrelevant goat antibody/Protein A agarose was assessed in the absence of tTG (lane 1).

tTG cDNA fused to the GAL4 DNA-binding domain in the pAS2-1 plasmid. For the two-hybrid screen, the recombinant plasmids were co-transformed into a reporter yeast strain Y190, which possesses the HIS3 and lacZ genes under control of a GAL4 responsive element. Co-transformants were plated on yeast drop-out media lacking leucine, tryptophan and histidine to assay for activation of the HIS3 reporter gene. This allowed for a positive growth selection of clones that were transformed by the two interacting hybrid constructs. Positive β-Galactosidase staining of the clones allowed further confirmation of the interaction.

Screening of the human liver cDNA library identified a number of interacting clones. The specificity of interaction with tTG was confirmed by bait plasmid loss and by the absence of transactivation with non-specific baits. Positive clones were mated with clones of the Y187 yeast strain containing the following non-specific DNA-BD constructs: (1) non-recombinant pAS2-1, (2) recombinant pAS2-1 encoding for bait fusion, and (3) an unrelated fusion (the control plasmid pLAM 5′-1, Clontech). Diploids from the mating were screened for β-Galactosidase expression. Positive clones that specifically interacted only with recombinant pAS2-1/tTG fusion plasmid, but not with three other plasmids, were isolated.

The sequences of the cDNA from these clones were compared with the nucleotide sequences of known proteins using the BLASTN search algorithm. Nucleotide sequences of the two positive clones (5-3 and 6-1) obtained from two separate screenings demonstrated remarkable homology to nucleotide and amino acid sequences of human importin-α3 (Qip-1) subfamily proteins, importin-α3 and importin-α4, with amino acid sequence identity of 100 and 93% respectively ($P = 3e^{-98}$) by BLASTX. The region of homology spans 152–332 amino acids, which encompass arms 2 to 6 of eight importin-α3 'armadillo' repeats that are responsible for direct binding of NLS [18]. Importin-α proteins belong to three subfamilies, of which importin-α3 and importin-α4 constitute the importin-α3/Qip-1-like subfamily [19]. Amino acid sequence identity of clones 5-3 and 6-1 with other proteins of the importin-α family, importin-α1 and α2, was 48 and 53%, respectively.

Analysis of the primary sequence of tTG suggested the presence of a putative bipartite NLS with high homology to the NLS of the NS1 non-structural protein of influenza virus [20]. The first site (DILRR) is located at position 259–263 and bears 80% homology to the NLS of the NS1 non-structural protein of influenza virus [20], and the second (PKQKRK) is located at position 597–602, and is identical to the second

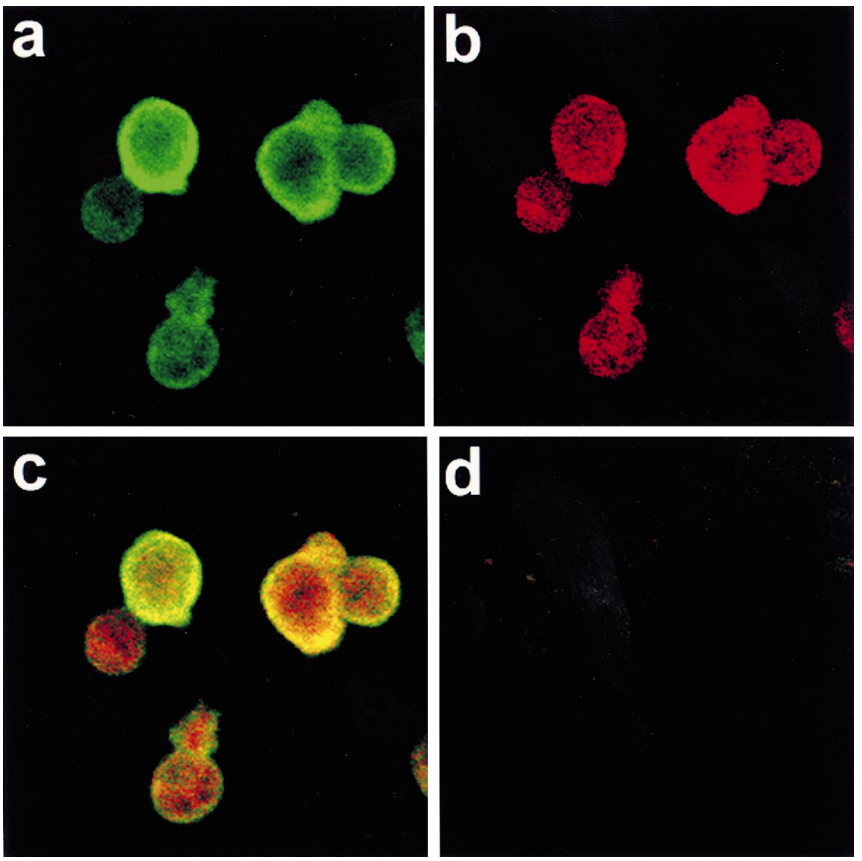

Fig. 3. Immunofluorescent images showing intracellular distribution of tTG and importin-α3 in NCI-H596 cells. NCI-H596 cells were stained with specific polyclonal goat anti-tTG antibody (A, green) followed by staining with specific polyclonal rabbit anti-importin-α3 antibody (B, red). Superimposition of the images (C, yellow) demonstrates the co-localization of tTG and importin-α3 in the cytoplasm of NCI-H596 cells. For negative control cells were double-stained with irrelevant isotype matched antibodies (D for superimposed image).

NLS of the same viral protein (Fig. 1). Further experiments are underway to confirm the functional significance of bipartite NLS on tTG.

3.2. *In vitro and in vivo interaction of importin-α3 with tTG*

To confirm the importin-α3/tTG binding in vitro we have demonstrated that in vitro translated [^{35}S]methionine-labeled importin-α3 was able to bind to human tTG immobilized on Protein A/G agarose. No binding was observed when irrelevant antibody was used as a control for specificity (Fig. 2).

To determine whether importin-α3 interacted with tTG in live mammalian cells, we used human non-small cell lung cancer cell line, NCI-H596 with high expression of tTG [21]. We performed dual immunohistochemical staining of NCI-H596 cells with anti-tTG and anti-importin-α3 antibodies (Fig. 3). tTG was localized predominantly in the cytosolic fraction with lower but detectable staining in the nuclei, whereas importin-α3 was equally expressed in cytosolic and nuclear regions. The uniform pattern of intracellular distribution of importin-α3 agrees with other published observations and is due to the rapid shuttling of importin-3α between the nucleus and the cytoplasm [22]. Co-localization of tTG and importin-α3 could be detected in the cytosol indicating the possible physiological association of the two molecules in vivo. Such co-localization pattern also agrees with the known mechanisms of importin-mediated nuclear transport, whereby importin-α molecule remains bound to a transported protein only in the cytosol and rapidly dissociates from it after crossing the nuclear membrane [19,23].

To further confirm the in vivo association of tTG with importin-α3, we performed co-immunoprecipitation of the two proteins from NCI-H596 cell extracts. When importin-

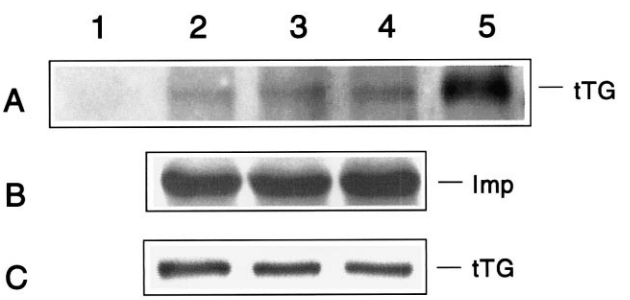

Fig. 4. Co-immunoprecipitation of tTG with antibody against importin-α3 in cultured NCI-H596 cells. NCI-H596 cells were treated with 10^{-5} M RA for 0–6 h. Importin-α3 was immunoprecipitated from the cytosolic fraction using polyclonal rabbit anti-importin-α3 antibody, and tTG (A) or importin-α3 (B) were detected in immunoprecipitates by Western blots as described in Section 2. tTG was detected in cytosolic extracts of RA-treated NCI-H596 cells (C). Immunoprecipitation with irrelevant IgG, negative control (lane 1); 0, 3 and 6 h of incubation with RA (lanes 2, 3 and 4); total cytosolic tTG, positive control (lane 5). Band densities were determined by scanning densitometry and normalized to control.

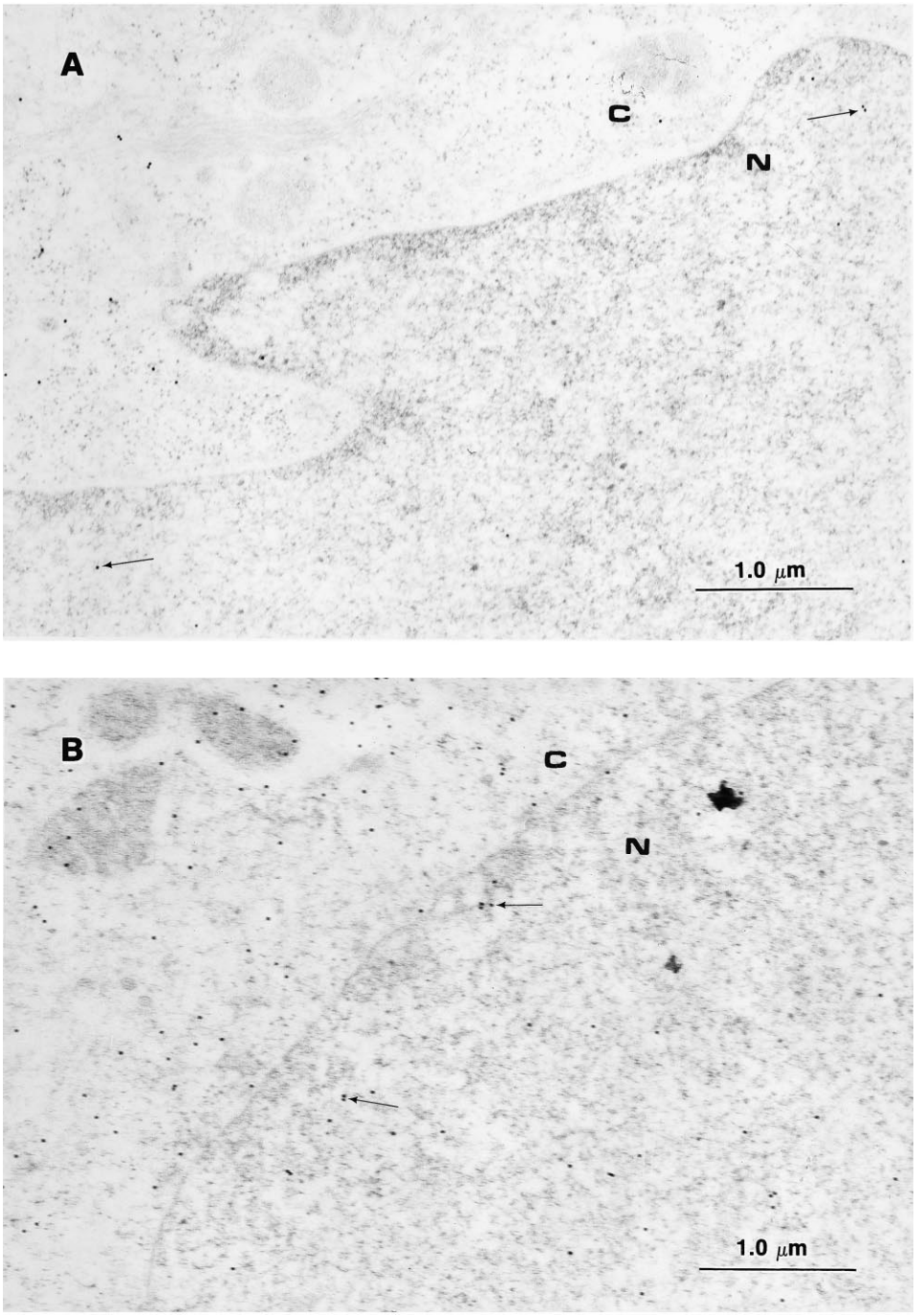

Fig. 5. Electron microscopic detection of tTG in NCI-H596 cells using immunogold staining. A: untreated cell; B: RA-treated cell. N, nucleus; C, cytosol; arrow indicates a gold particle; bar indicates 1 μm. At least 10 cells were examined in each group. No nuclear staining was observed when monoclonal antibody against keratinocyte TG (BC.1) was used as a negative control (not shown).

α3 was immunoprecipitated and immunoblotted with anti-tTG antibody, the positive staining of an 85 kDa band was observed (Fig. 4A). However, we were unable to detect importin-α3 signal when tTG was precipitated using the anti-tTG goat polyclonal antibody or Cub 7401 monoclonal antibody, followed by immunodetection with anti-importin-α3 antibody (data not shown). Since en route to the nucleus tTG may form a complex with importin-α and -β, it is possible that in such a complex the anti-tTG antibody recognition site is not exposed. Another explanation is that tTG:importin stoichiometry in cells is not favorable for bringing down detectable amounts of importin-α3 when tTG is immunoprecipitated.

3.3. Nuclear expression of tTG in NCI-H596 cells by electron microscopy

Nuclear expression and activity of tTG both as a G-protein and transamidase have been recently established [14,15]. We examined nuclear localization of tTG in NCI-H596 cells stimulated with all-trans retinoic acid (RA), which is known to upregulate tTG and induce apoptosis in many cell types including NSCLC [24,25]. To determine if tTG is localized to

the nucleus in NCI-H596 cells we immunodetected intracellular tTG by electron microscopy using Cub 7401 monoclonal antibody and immunogold-conjugated secondary antibody (Fig. 5). By electron microscopy, nuclear tTG expression was low in unstimulated NCI-H596 cells (0.3 ± 0.03 gold particles/μm^2), but was markedly upregulated after 24 h of incubation with 10^{-5} M RA (4.5 ± 1.29 gold particles/μm^2). Although by 24 h RA upregulates protein expression of cytosolic tTG in NCI-H596 [25] this alone can not explain the increased expression of nuclear tTG since the high molecular weight of this protein, 80 kDa, precludes the passive diffusion of tTG through the nuclear membrane. In fact, upregulation of nuclear tTG by RA in NCI-H596 cells was preceded by a slight (1.4-fold by scanning densitometry) but statistically significant ($P \leq 0.01$) increase in tTG/importin-α3 co-immunoprecipitation after 6 h of incubation (Fig. 4A) implicating active nuclear transport in RA-challenged NSCLC cells. RA did not alter the expression of importin-α3 or cytosolic tTG by 6 h of incubation (Fig. 4B, C).

Our data demonstrate that a nuclear transport protein, importin-α3, is an intracellular tTG-binding counterpart. Although the possibility that importin-α3 is a mere target of tTG transamidase activity cannot be excluded, our data and previous observations are consistent with importin-α3 being involved in the active transport of tTG into the nucleus. Discovery of the tTG/importin interaction was unexpected, but not surprising. Several pieces of evidence, including the data presented in this report, indicate the nuclear expression of tTG [14,15]. Considering the high molecular weight of this protein, 80 kDa, active nuclear transport is the only means to translocate this molecule through the nuclear membrane. The role of nuclear tTG has not been addressed so far. This study indicates that RA, which has been shown to induce apoptosis in NSCLC cells specifically upregulates nuclear tTG expression. An intriguing possibility is that nuclear tTG activity is responsible for the known roles of tTG in cell cycle progression and apoptosis. Identification of nuclear histones and Rb protein as the tTG substrates suggested the potential mechanisms of such roles.

In conclusion, results from this study suggest that tTG may be actively transported into the nucleus via binding to an importin-α3/Qip-1 family protein. Experiments are in progress to define the NLS within tTG by site-directed mutagenesis. Further studies are underway to confirm the functional relevance of importin-α binding and nuclear transport of tTG in cellular processes.

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