Identification of the Eukaryotic Initiation Factor 5A as a Retinoic Acid-stimulated Cellular Binding Partner for Tissue Transglutaminase II*

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GTP-binding protein/transglutaminases (tissue transglutaminases or TGases) have been implicated in a variety of cellular processes including retinoic acid (RA)-induced apoptosis. Recently, we have shown that RA activates TGases as reflected by stimulated GTP binding, increased membrane association, and stimulated phosphoinositide lipid turnover. This prompted us to search for cellular proteins that bind TGases in a RA-stimulated manner. In this report, we show that the eukaryotic initiation factor (eIF-5A), a protein that is essential for cell viability, perhaps through effects on protein synthesis and/or RNA export, associates with the TGase in vivo. The interaction between eIF-5A and TGase is specific for the GDP-bound form of the TGase and is not detected when the TGase is pre-loaded with GTPγS. The TGase-eIF-5A interaction also is promoted by Ca2+, Mg2+, and RA treatment of HeLa cells. In the presence of retinoic acid, millimolar levels of Ca2+ are no longer required for the TGase-eIF-5A interaction. No-codazole treatment, which blocks the cell cycle at mitosis (M phase), strongly inhibits the interaction between eIF-5A and cytosolic TGase. The interaction between TGase and eIF-5A and its sensitivity to the nucleotide-occupied state of the TGase provides a potentially interesting connection between RA signaling and protein synthesis and/or RNA trafficking activities.

Tissue transglutaminases (TGases) represent an interesting class of enzymes that were originally identified based on their ability to catalyze the Ca2+-dependent formation of covalent bonds between peptide-bound glutamyl residues and the primary amino groups in a variety of compounds (1). It was then realized that these enzymes are regulated by GTP and in fact can undergo a GTP-binding/GTPase cycle like classical G proteins (2–4). More recently, it also has been appreciated that these enzymes are capable of ATP hydrolysis at a site distinct from the GTP-binding site (5). The TGases appear to be widely distributed within the cell, mainly present in the cytosol (6, 7), but are also found in plasma membranes (8) and in the nucleus (9). They have been implicated in a variety of fundamentally important cellular functions, including cell adhesion, wound healing, and cellular differentiation and apoptosis (10–13). It seems likely that the TGases play a role as signaling transducers in some of these biological responses (again reminiscent of classical G proteins), with one particular example being the mediation of α1-adrenergic receptor regulation of phosphoinositide lipid turnover through the ability of the GTP-bound TGase to stimulate phospholipase C-ε (14).

Our laboratory has been interested in further probing the possible importance of the GTP-binding/GTPase cycle of TGases in their biological function. Recently, we found that retinoic acid (RA) treatment of HeLa cells under conditions that give rise to cellular differentiation and apoptosis strongly stimulated the GTP binding activity of the TGases (15). This was accompanied by an increased association of TGase with the plasma membrane and a concomitant stimulation of phospholipase C activity. These RA-stimulated effects could not be attributed to changes in the expression of the TGase, which caused us to propose that exposure of cells to RA may result in the expression of specific regulatory factors for the TGase, e.g., lipid-modifying enzymes and/or other proteins capable of imparting posttranslational modifications such as protein kinases.

In the present study, we set out to extend this work and identify possible regulatory factors as well as other potential target/effecter molecules for the TGase. In particular, we followed up on an observation that an ~18-kDa protein appeared to be co-purifying with TGase from rabbit liver preparations. We have identified this protein as the eukaryotic initiation factor (eIF-5A), which was originally suspected to play an important role in protein synthesis but more recently has also been suggested to participate in nuclear (RNA) export (16). We show here that eIF-5A is a specific cellular binding partner for a particular form of the TGase and that this interaction is stimulated by RA, thus suggesting an interesting and potential link between protein synthesis and/or RNA trafficking and RA regulation of cellular activities.

EXPERIMENTAL PROCEDURES

Construction of a Recombinant Baculovirus to Express Human Transglutaminase in Spodoptera frugiperda Sf21 Cells—The cDNA clone of human tissue TGase was kindly provided by Dr. Peter Davies (University of Texas Medical School, Houston) in a pSG5 vector (Stratagene). It was digested with NcoI, and the resultant 2.2-kilobase fragment, including the coding region of the protein, was ligated into pGEXKG-lin (17), which was predigested with NcoI so that the TGase gene was fused in-frame downstream of the glutathione S-transferase (GST) coding region of the vector. The resultant construct was designated pGEXKG-lin-TG. The XbaI fragment of the pGEXKG-lin-TG containing the GST-tagged TGase was further subcloned into the baculovirus expression vector pVL1393 at the XbaI site.

The baculovirus expression vector containing GST-TGase was co-transfected with BaculoGold DNA into Sf21 cells using the BaculoGold transfection kit (Pharmering, San Diego, CA). Recombinant viruses were isolated by plaque purification (18). Sf21 cells were infected by recombinant viruses at a multiplicity of infection of 5 plaque forming units. This paper is available on line at http://www.jbc.org

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1 The abbreviations used are: TGases, transglutaminases; GTPγS, guanosine 5’-3-O-(thiotriphosphate); RA, retinoic acid; eIF-5A, eukaryotic initiation factor-5A; GST, glutathione S-transferase; DTT, dithiothreitol; TTBS, Tris-buffered saline, 0.2% Tween 20; PAGE, polyacrylamide gel electrophoresis.
units/cell. The infected cells were harvested 60 h postinfection, and the cell pellets were stored frozen at −80 °C.

**Protein Purification of TGase from SF21 Cells**—The pellets were thawed (50% v/v) in buffer A (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 100 μM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin at 4 °C for 1 h. The suspension was centrifuged at 12,000 × g for 1 h, and the supernatant was mixed with glutathione-agarose beads (10% v/v) and incubated at 4 °C by continuous shaking for 1 h. The suspension was then centrifuged at 100,000 × g for 10 min, and the beads were washed three times with buffer A and suspended in the same buffer (50% v/v) for storage.

**Cell Culture**—HeLa cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM l-glutamine, 100 IU/ml of penicillin and streptomycin, 2.0 g/liter sodium bicarbonate, and 10 mM HEPES in a humidified atmosphere with 5% (v/v) CO₂ at 37 °C. To achieve cell-cycle arrest in M phase, nocodazole (80 μg/ml) was added to the growth medium, and cells were allowed to grow for 24 h. For harvesting, the cells were trypsinized and suspended with RPMI 1640 containing 10% fetal calf serum.

**Cell Fractionation**—Cells were pelleted and washed twice in ice-cold hypotonic buffer containing 10 mM HEPES, pH 8.0, 5 mM KCl, and 2 mM MgCl₂. The cell pellets were then quick frozen in liquid nitrogen for storage. Thawed cells were resuspended in buffer B (25 mM HEPES, pH 7.4, 25 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 μM phenylmethylsulfonyl fluoride, 0.2 mM benzamidone, and 10 μg/ml aprotinin and leupeptin each). The suspension was homogenized using a Potter-Elvehjem homogenizer. Cellular debris was spun out at 3000 × g for 15 min. The supernatant was collected and spun at 100,000 × g for 45 min. Nonidet P-40 was added to the supernatant to a final concentration of 1% (this was used as a cytosolic fraction), and the membrane pellet was resuspended in 1% Nonidet P-40 in 25 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2 mM benzamidone, 100 μM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin and leupeptin each.

**Immunoprecipitations and Precipitation with Glutathione-Agarose**—For the precipitation of eIF-5A by the GST-TGase fusion protein, the clear supernatant representing the cytosolic fraction of HeLa cells was transferred to fresh microcentrifuge tubes. It was mixed with a suspension (25 μl) of GST-TGase beads and incubated for 2 h at 4 °C in the presence of different concentrations of salts (CaCl₂ and MgCl₂) and nucleotides (GDP and GTPγS) as indicated in the figures. The suspension was centrifuged, and the pellet was washed three times with buffer A and finally suspended in two times Laemmli sample buffer (19) and boiled for 5 min. The supernatant was used for the purpose of electrophoresis and Western blotting.

For the immunoprecipitation of eIF-5A, supernatants (200 μl) were incubated for 1 h at 4 °C with 5 μl of rabbit anti-eIF-5A polyclonal antibody, kindly provided by Dr. Edith Wolff (NIH, Bethesda, MD). Protein A-Sepharose (5 mg/ml lysate) was then added to the sample and incubated for 1 h at 4 °C. A parallel set of control samples containing Protein A-Sepharose (5 mg/ml lysate) was then added to the sample and antibody, kindly provided by Dr. Edith Wolff (NIH, Bethesda, MD).

**RESULTS AND DISCUSSION**

Various lines of evidence have suggested that GTP-binding protein/transglutaminases (TGases) may represent a new family of transducer molecules that upon binding GTP can stimulate target/effecter activities such as phosphoinositide lipid turnover (8, 20). We recently reported that a number of biochemical activities of TGases are stimulated by RA including GTP binding (and the resultant GTP hydrolytic activity), transglutaminase activity, plasma membrane-association and phosphoinositide lipid turnover (15). The regulatory mechanisms underlying these RA-stimulated activities are not known, although they cannot be attributed to simple changes in the levels of TGase expression (15). As a means to gain further insight into the interplay between RA and TGases, as well as into the function of TGases, we have set out to identify potential regulators and other binding partners or targets for these GTP-binding proteins.

Based on an earlier observation that an ~18-kDa protein appeared to co-purify with TGases from rabbit liver,² we obtained sufficient amounts of the 18-kDa protein for microsequencing analysis and determined that it was the eukaryotic initiation factor-5A (eIF-5A). At present, little is known regarding the cellular functions of eIF-5A. It has been suggested to participate in some aspect of protein synthesis at the ribosomal subunit-joining step and/or at a later stage of 80 S ribosomal

² U. S. Singh, unpublished results.
initiation complex formation (21). Recently, eIF-5A also has been suggested to play a role in RNA export, based on its interaction with the HIV-1 protein, Rev (16). The eIF-5A protein is unique in that it is the only cellular protein known to contain the unusual amino acid hypusine; a number of lines of evidence indicate that hypusine and eIF-5A are essential for the interaction also required both Ca

We next examined whether the TGase-eIF-5A interaction occurred in intact cells. Using an anti-eIF-5A polyclonal antibody that can immunoprecipitate eIF-5A from cells (Fig. 2, top panel), we found that TGase was specifically co-precipitated with eIF-5A (Fig. 2, middle panel, lanes 3 and 4) but not with nonimmune IgG (Fig. 2, middle panel, lanes 1 and 2) as indicated by Western blotting with an anti-TGase antibody. The TGase that co-precipitates with eIF-5A can also be photoaffinity labeled with \([\alpha-\beta^3P]GTP\) (Fig. 2, lower panel, lanes 3 and 4).

The ability of the eIF-5A-associated TGase to bind GTP indicates that it belongs to an activated pool of the endogenous TGase. Previously, we had shown that in HeLa cells most of the TGase was inactive and not capable of binding \([\alpha-\beta^3P]GTP\) (15). However, as alluded to earlier, RA, which causes the cells to differentiate and undergo apoptosis (13), strongly stimulated the GTP binding activity of the cellular TGase (15). Since this
we therefore reasoned that if it was the activated pool of TGase capable of GTP binding activity and an increased ability to TGase, thus creating an activated pool of protein that was in the eIF-5A precipitates. Fig. 3 (a very brief exposure (detected in co-immunoprecipitates with eIF-5A even after only specific anti-TGase antibody (i.e., a protein kinase) that modified the TGase, thus creating an activated pool of protein that was capable of GTP binding activity and an increased ability to associate with the plasma membrane. Given this suggestion, we therefore reasoned that if it was the activated pool of TGase that associates with eIF-5A, then RA treatment of cells should stimulate the formation of a TGase:eIF-5A complex. The results presented in Fig. 3 show that this in fact is the case. Specifically, following RA treatment of HeLa cells, TGase was detected in co-immunoprecipitates with eIF-5A even after only a very brief exposure (~2 min) of the blot and under conditions where in the absence of RA, little detectable TGase was present in the eIF-5A precipitates. Fig. 3 (right panel) also shows that RA treatment strongly increased the amount of [α-32P]GTP binding activity (corresponding to the TGase) that co-immunoprecipitated with eIF-5A.

We have been able to further probe the connection between RA-promoted activation of TGase and its interaction with eIF-5A by taking advantage of a recent finding that nocardazole treatment of HeLa cells (under conditions that cause the cells to accumulate in M phase) inhibits the ability of membrane-associated TGase to bind GTP and thus uncouples the ability of the RA-regulated TGase to associate with plasma membranes from its ability to become activated (data not shown). It is interesting that nocardazole treatment also strongly inhibits the association of the cytosolic TGase with eIF-5A. The results presented in Fig. 4 clearly show that the co-precipitation of TGase with eIF-5A does not occur in HeLa cells that have been pretreated with nocardazole, either when monitoring this interaction by Western blotting with the specific anti-TGase antibody (upper panel) or by photoaffinity labeling with [α-32P]GTP (lower panel). A similar inhibitory effect by nocardazole was also obtained with RA-treated HeLa cells (data not shown).

The results presented in Fig. 5 show that the effects of both RA and nocardazole treatment are directed at eIF-5A as well as at the membrane-bound pool of TGase. Given that Ca2+ appeared to serve as a necessary cofactor for TGase-eIF-5A interactions (see Fig. 1), we performed an experiment to determine whether treatment with RA or nocardazole influenced the ability of Ca2+ to stimulate these interactions. Specifically, lysates were prepared from control HeLa cells and from RA-treated cells or nocardazole-treated cells and then incubated with the recombinant GST-TGase in the presence of different levels of CaCl2. The GST-TGase-eIF-5A complexes were precipitated with glutathione-agarose and Western blotted with the anti-eIF-5A polyclonal antibody. As shown in Fig. 5, the GST-TGase-eIF-5A interaction was maximal when lysates from control cells were incubated with 1 mM CaCl2, whereas in RA-treated cells GST-TGase-eIF-5A interactions were clearly detected even in the absence of added CaCl2. These results suggest that RA treatment has a direct effect on eIF-5A, such that it is able to associate with the TGase even at low (micromolar) levels of Ca2+. It should be noted that nocardazole treatment completely eliminated the interaction between GST-TGase and eIF-5A. Because nocardazole did not decrease the levels of eIF-5A detected in these lysates by Western blotting (data not shown), these findings suggest that the nocardazole effects on TGase-eIF-5A interactions are directed at eIF-5A, such that it cannot bind to the TGase even in the presence of millimolar levels of Ca2+. Thus, like RA, nocardazole exhibits regulatory effects on both partners of the TGase-eIF-5A binding interaction.

These results when taken together with previous findings are consistent with the following model (Fig. 6). Treatment of HeLa cells with RA promotes the formation of an activated pool of the TGase, which has an increased affinity for the plasma membrane and the capability to undergo GTP/GDP exchange. That portion of the membrane-associated TGase that binds GTP can then stimulate the activity of a phospholipase C enzyme (15); recent work would suggest that this is the phospholipase-C1 isoform (14). The TGase-promoted stimulation of phospholipase C activity gives rise to an increase in intracellular Ca2+ which has been suggested to stimulate the enzymatic transglutaminase activity of the TGase (29). If Ca2+ binding to the TGase has some type of regulatory affect on the transglutaminase active site, such an effect might account for the ability of Ca2+ to promote TGase-eIF-5A interactions. The involvement of the transglutaminase active site in binding
EIF-5A also might explain an earlier observation that the unique hypusine residue of EIF-5A serves as substrate for transglutaminase reactions, i.e. it can be cross-linked to the \( \gamma \)-carboxamide group of glutamine side chains (30). Within this scheme, RA could promote TGase-EIF-5A interactions both by initiating a cascade of events that results in the membrane association of TGase and a resultant stimulation of phospholipase C activity and increase in intracellular \( \text{Ca}^{2+} \), and by having an as yet undetermined effect on the EIF-5A molecule (perhaps by influencing some type of posttranslational modification), such that EIF-5A can bind the TGase at micromolar levels of \( \text{Ca}^{2+} \). Nocodazole treatment by inhibiting the activation of membrane-associated TGase, as well as by exerting a regulatory effect on EIF-5A, inhibits TGase-EIF-5A interactions.

Clearly, a critical question concerns how the TGase-EIF-5A interaction influences the normal functions of these proteins. We have not found any effect on the various measurable TGase activities; however, as alluded to earlier, an interesting possibility is that TGases influence the cellular localization of EIF-5A. When the TGase is in a GDP-bound state (following GTP hydrolysis (31)), it can be bound by EIF-5A and maintain EIF-5A in the cytoplasm. However, upon GTP-GDP exchange, the EIF-5A is released perhaps allowing the molecule to bind to some other cytosolic (target) protein involved in protein synthesis and/or to return to the nucleus to participate in some aspect of RNA trafficking. Thus, the GTP-binding/GTPase cycle of the TGase could serve as a timing device to regulate changes in the cellular localization of EIF-5A that are important for either protein synthesis or RNA trafficking between the cytoplasm and the nucleus. The fact that nocodazole treatment perturbs both the GTP-binding/GTPase cycle of membrane-associated TGase, and the ability of EIF-5A to associate with the TGase, further suggests that EIF-5A activity might be coupled to the cell cycle. Taken together, these results would suggest that other factors that influence cell-cycle progression may also exert regulatory effects on TGase-EIF-5A interactions. Future studies will be directed toward testing the different possibilities raised above and determining the identity of additional cellular factors that mediate the regulation of TGase activities by RA as well as additional binding partners for EIF-5A.

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