Phosphoinositide 3-Kinase Activity Is Required for Retinoic Acid-induced Expression and Activation of the Tissue Transglutaminase*

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Tissue transglutaminase (TGase) is a dual function enzyme that couples an ability to bind GTP with transamidation activity. Retinoic acid (RA) consistently induces TGase expression and activation, and it was recently shown that increased TGase expression protected cells from apoptosis. To better understand how RA regulates TGase, we considered whether RA employed pro-survival signaling pathways to mediate TGase expression and activation. It was found that RA stimulation of NIH3T3 cells activated ERK and phosphoinositide 3-kinase (PI3K); however, only PI3K activation was necessary for RA-induced TGase expression. The overexpression of a constitutively active form of PI3K did not induce TGase expression, indicating that PI3K signaling was necessary but not sufficient for TGase expression. The exposure of cells expressing exogenous TGase to the PI3K inhibitor, LY294002, reduced the ability of TGase to be photoaffinity-labeled with [α-32P]GTP, providing evidence that PI3K regulates the GTP binding activity of TGase as well as its expression. Moreover, cell viability assays showed that incubation of RA-treated cells with LY294002 together with the TGase inhibitor, monodansylcadaverine (MDC), converted RA from a differentiation factor to an apoptotic stimulus. These findings demonstrate that PI3K activity is required for the RA-stimulated expression and GTP binding activity of TGase, thereby linking the up-regulation of TGase with a well established cell survival factor.

Tissue transglutaminase (TGase) is a unique dual function protein that contains an enzymatic transamidation activity and a GTP-binding capability similar to other classical G-proteins (1). The transamidation activity of the TGase catalyzes a reaction in which donor glutamine residues from proteins are covalently linked to acceptor primary amino groups of other proteins or polyamines. The generation of these protein linkages is believed to modulate a variety of cellular processes including the maintenance of extracellular matrix, endocytosis, differentiation, and apoptosis (2–6). Moreover, aberrant TGase enzymatic activity has been linked to several neurodegenerative disorders (1), indicating that proper regulation of TGase activity is required for normal cellular functions.

One way in which TGase activity is managed is through the regulation of its expression. Many cell types express the TGase protein at low levels, and increases in its expression often occur only after prolonged exposure to certain stimuli (7, 8). A consistent inducer of TGase expression is retinoic acid (RA) (8–12), which imparts its cellular effects by binding to a family of retinoic acid receptors (RARs). Retinoic acid-bound RARs function as transcriptional activators that bind to RA response elements found in the promoters of various genes (13, 14). Deletion analysis of the promoter of the TGase gene identified two RA response element motifs, which were required for RA-induced TGase transcription (15). Other transcriptional regulators are thought to influence TGase expression (16), but the identity of these factors and their importance for RA-mediated TGase expression are unclear.

Once TGase is expressed, its enzymatic activity can be regulated by cofactors such as GTP and calcium (Ca2+). Studies have demonstrated that GTP-bound TGase was limited in its ability to catalyze transamidation, whereas GTP hydrolysis and the binding of Ca2+ enhanced transamidation activity (17, 18). However, others have reported that both GTP and GDP inhibit the transamidation activity of the TGase (19), suggesting that GTP binding is not a specific regulator of transamidation but rather may primarily serve to transduce signals. For example, the α2-adrenergic receptor-mediated stimulation of phospholipase C activity was shown to be mediated by an 80-kDa GTP-binding protein, which was later identified as TGase (20).

Here we used RA-induced TGase expression and activation as a model system to better understand how TGase is regulated in cells. Because we recently reported that TGase activity was implicated in cell survival (7), we considered whether RA utilizes pro-survival pathways to mediate TGase expression or activation. We found that both phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) were activated by RA, but only PI3K activity was necessary for RA to induce TGase expression. The inhibition of PI3K or TGase activity converted RA from a differentiation factor into an apoptotic signal, suggesting that the survival effect of PI3K may at least in part be attributed to its ability to up-regulate TGase. We further determined that GTP-binding to the TGase was dependent on PI3K. To our knowledge, these findings provide the first demonstration that the induction of TGase expression and GTP binding activity by RA requires the pro-survival factor, PI3K.

EXPERIMENTAL PROCEDURES

Materials—PD98059 and LY294002 were purchased from Calbiochem, and EGF and LipofectAMINE PLUS transfection reagent were

*The work was supported in part by National Institutes of Health Postdoctoral Grant F32 GM208052 (to M. A.) and Grant NIH R01 GM61762 (to R. A. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1The abbreviations used are: TGase, tissue transglutaminase; RA, retinoic acid; RAR, retinoic acid receptor; PI3K, phosphoinositide 3-kinase; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; HPR, all-trans-4-hydroxyphenyllretinamide; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; AMP-PNP, adenosine 5′-o-[β,γ-imino]triphosphate; HA, hemagglutinin; MDC, monodansylcadaverine.
NIH3T3 cells were maintained in Dulbecco containing 10% fetal bovine serum and 100 units/ml penicillin, and containing 10% calf serum with 100 units/ml penicillin. Both cell lines stated.

Sciences, and all other materials were from Fisher unless otherwise stated.

X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% NaF, 1 mM sodium (1% calf serum). The following day, for 2 days were placed in low serum media (5% calf serum). The filters were incubated with the various primary antibodies was used to measure the activities of ERK and AKT. The blots were stripped and then reprobed with antibodies that recognized total TGase, ERK, and AKT to assess the expression levels of each of these proteins. B, whole cell lysates of cells treated without (−) or with RA were affinity-labeled with radioactive GTP and then immunoprecipitated with a TGase antibody (L.P. TGase) or an ERK antibody (L.P. ERK). SDS-PAGE and transfer to nitrocellulose membrane were performed on the immunoprecipitations, and the membrane was exposed to film to monitor the photoincorporation of [32P]GTP by TGase. Expression of the TGase was detected by probing the membrane with a TGase antibody (WB: TGase).

obtained from Invitrogen. RA, all-trans-N-(4-hydroxyphenyl)retinamide (HPR), and monodansylcadaverine (MDC) were obtained from Sigma. The anti-TGase antibody was from Neomarkers, the anti-HA antibody was from Covance, and the anti-phospho-ERK, anti-ERK, anti-active AKT, and anti-AKT antibodies were all obtained from New England Biolabs. [α-32P]GTP was purchased from PerkinElmer Life Sciences, and all other materials were from Fisher unless otherwise stated.

Cell Culture—HL60 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 100 units/ml penicillin, and NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% calf serum with 100 units/ml penicillin. Both cell lines were maintained in a humidified atmosphere with 5% CO2 at 37°C. For the various treatments described, the cells were grown to confluence in medium containing 10% serum, and then medium containing 1% serum with 5 μM RA or HPR or 100 ng/ml EGF, ±6 μM LY294002, or 10 μM PD98095 was added for the times indicated under “Results and Discussion.” Cells were rinsed with phosphate-buffered saline and then lysed with cell lysis buffer (10 mM NaHPO4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% NaF, 1 mM NaVO4, 25 mM β-glycerophosphoric acid, 100 μM/ml phenylmethylsulfonyl fluoride, and 1 μg/ml each of aprotinin and leupeptin, pH 7.35). The lysates were clarified by centrifugation at 12,000 × g for 10 min at 4°C. Protein concentrations were determined using the Bio-Rad DC protein assay.

Western Blot Analysis—Total cell lysate of each sample was combined with Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE for the resolution of each protein analyzed. The proteins were transferred to nitrocellulose filters and blocked with TBST (20 mM Tris, 137 mM NaCl, pH 7.4, and 0.02% Tween 20) containing 5% nonfat dry milk. The filters were incubated with the various primary antibodies diluted in TBST for 2 h at room temperature and then washed three times with TBST. To detect the primary antibodies, anti-mouse or rabbit conjugated to horseradish peroxidase (Amersham Biosciences) diluted 1:5000 in TBST was incubated with the filters for 1 h, followed by three washes with TBST. The protein bands were visualized on x-ray film after exposing the filters to chemiluminescence reagent (ECL, Amersham Biosciences).

Photoaffinity Labeling of the TGase—Photoaffinity labeling of the TGase was performed by incubating whole cell lysates with 3 μCi of [α-32P]GTP in 50 mM Tris-HCl, pH 7.4, 2 mM EGFTA, 1 mM dithiothreitol, 20% (w/v) glycerol, 100 mM NaCl, and 500 μM AMP-PNP for 10 min at room temperature. The samples were placed in an ice bath and irradiated with UV light (254 nm) for 15 min, mixed with 5× Laemmli sample buffer, and boiled for 5 min. SDS-PAGE was performed followed by transfer to nitrocellulose filters and exposure on x-ray film.

RESULTS AND DISCUSSION

Several studies have demonstrated that the expression and activation of the TGase are tightly coupled to the effects of RA (8–12). However, it is unclear whether the increases in TGase protein levels and GTP binding activity induced by RA occur solely through up-regulation of the transcriptional activities of the RARα or whether the activation of additional signaling components is needed. Because we recently implicated TGase as a survival factor (7), we were interested in determining whether the anti-apoptotic molecules PI3K and ERK influence TGase expression and/or activation.

Fig. 1A shows that when NIH3T3 cells were incubated with RA there was a progressive increase in TGase protein expression as determined by Western blot analysis and GTP binding activity. GTP binding activity was measured by the incorporation of [α-32P]GTP into an ~80-kDa protein, which we have verified to be TGase based on both protein purification (18) and immunoprecipitation with an anti-TGase antibody (Fig. 1B, for review see Ref. 21). Consistent with earlier findings that showed that long term treatments were required for the up-regulation of TGase activity (1, 7, 8), nearly 24 h of RA stimulation were needed before increases in TGase expression and
activation were evident (Fig. 1A). We then measured the ability of RA to activate ERK using an antibody that recognizes only the phosphorylated forms of ERK1/2. Within 5 min of exposure to the retinoid, ERK activity could be detected at a level comparable with that observed with the positive control, EGF. The RA-induced ERK activation was sustained up to 1 h and then reduced to near basal activity for the duration of the experiment. From the same cell lysates used to analyze ERK induction, PI3K activity was also examined using Akt phosphorylation or activation as a readout. The phosphorylation of Akt provides a reliable assay for PI3K activation, because it has been well established that several pro-survival signals up-regulate Akt phosphorylation in a PI3K-dependent manner (22, 23). Utilizing an Akt phospho-specific antibody, we found that Akt phosphorylation was augmented by RA in a time-dependent fashion. Akt induction coincided with RA-mediated ERK activation, having maximal activation at 5 min and decreasing gradually to near background levels by 8 h of stimulation. Others have also demonstrated that ERK and PI3K activities were enhanced by RA in the human leukemia cell line HL60 (24, 25) and that inhibiting the activity of either kinase blocked RA-mediated differentiation. These findings indicate that retinoid-induced mitogen-activated protein kinase and PI3K activation are not unique to NIH3T3 cells and suggest that their signaling capabilities are involved in some aspects of RA-mediated cellular effects. The differences in the phosphorylation states of ERK and Akt detected with the phospho-specific antibodies throughout the duration of the RA time course were likely a result of posttranslational modifications (i.e. the activation of kinases and phosphatases) rather than changes in protein expression as the ERK and Akt protein levels remained constant in each sample. It is interesting that not only does RA up-regulate similar signaling pathways as growth factors, the rates of activation of ERK and Akt induced by RA closely parallel the transient activations of ERK and Akt following EGF stimulation (26, 27).

The fact that both ERK and PI3K activities appear to be enhanced by RA, which is coupled with the finding that RA induces TGase expression, raises the possibility that the activation of these kinases may play a role in up-regulating the expression of the TGase. To address this question, we took advantage of chemical inhibitors that specifically block the activations of either MEK, the immediate activator of ERK, or PI3K. NIH3T3 cells were stimulated with RA for 3 days with or without the addition of the MEK inhibitor, PD98059, or the PI3K inhibitor, LY294002, at concentrations known to inhibit the activity of each kinase (26), and the resulting expression and GTP binding activity of the TGase were determined. As shown in Fig. 2, left, incubating the cells with PD98059 appeared to have no effect on the ability of RA to induce TGase expression or GTP binding activity. Thus, the ERK pathway may be used by this retinoid to mediate the expression of some RA-responsive genes that are distinct from those that influence the expression or GTP binding activity of the TGase. In contrast, the exposure of RA-treated cells to LY294002 severely diminished the protein expression level and corresponding GTP binding activity of the TGase. In several experiments, the LY294002-mediated inhibition of TGase expression and activity ranged from 60 to 90% (Fig. 2 and data not shown). The overexpression of the p85 regulatory subunit of PI3K in cells also inhibited the RA-induced TGase expression and GTP binding activity similar to LY294002 (Fig. 3), further implicating PI3K signaling in the transcriptional regulation of the TGase. Reprobing the same blots from Fig. 2 with an antibody against actin showed equivalent amounts of this protein in each lane, indicating that the decrease in TGase expression by LY294002 was not a result of a general down-regulation in protein expression (Fig. 2, left). Similar experiments conducted on the human leukemia cell line, HL60, and several other cell types consistently found that RA-induced TGase expression was inhibited when the cells were co-incubated with the PI3K chemical inhibitor (Fig. 2, right and data not shown).

Because PI3K activation appears to be necessary for RA to up-regulate TGase expression, we next assessed whether continuous PI3K signaling would be sufficient to cause chronic TGase expression and/or activation in mouse fibroblasts. Despite the expression of a constitutively active form of PI3K (a myristoylated form of the p110 catalytic subunit) in fibroblasts, a corresponding increase in TGase expression was not evident (Fig. 3). Furthermore, the RA treatment of cells overexpressing myristoylated p110 up-regulated TGase expression and GTP binding activity to the same extent as control cells, indicating that continuous PI3K activation was not sufficient to sensitize the induction of TGase expression or activation by RA. Similar experiments conducted in NIH3T3 cells stably overexpressing dominant-active forms of the small G-proteins Ras, Rac, or Cdc42 also did not augment the basal level of RA-induced TGase expression or GTP binding (data not shown). We were also interested in determining whether factors that can activate PI3K other than RA might be capable of regulating TGase expression levels. Sustained stimulation of NIH3T3 cells with EGF for 2 days activated PI3K strongly as indicated by Akt phosphorylation (Fig. 1) but failed to produce a detectable increase in TGase protein levels (Fig. 3). These data strongly suggest that although PI3K activity is essential for RA to induce TGase expression, RA must also influence the function of an additional factor(s) in order to increase TGase expression. We expect that at least one of these factors is likely to be under the transcriptional control of a member of the RAR family based on two lines of evidence. First, the activation of this factor seems to be a specific outcome of RA signaling, because both RA and EGF stimulated PI3K activity but only RA was capable of augmenting TGase expression. Several studies have demonstrated that the RARs preferentially bind to and become activated by retinoids (13, 14). Second, it has been well established that the induction of the transcriptional activities of the RARs by binding RA is essential for retinoid-mediated TGase expression (15).
Regulation of TGase by PI3K

Fig. 3. Constitutive PI3K activity is not sufficient to induce chronic TGase expression or activation. Cells were transiently transfected with vector only, a HA-tagged myristoylated form of the catalytic subunit of PI3K (HA-p110M), or a HA-tagged form of the regulatory subunit of PI3K (HA-p85) and grown in complete growth medium for 1 day. The medium was replaced with low serum medium (5 μM RA), and the cultures were maintained for another 2 days and then lysed. SDS-PAGE and transfer to nitrocellulose membrane were performed on the whole cell extracts. The expression of the PI3K constructs was detected by probing the blot with a HA antibody, and the expression of TGase was detected using a TGase antibody. The whole cell extracts were also used in affinity labeling assays as outlined under “Experimental Procedures.” WB, Western blot.

Increases in TGase expression following exposure to RA are invariably coupled with the induction of the GTP binding activity of the TGase (Fig. 1) (7, 11). However, the molecular mechanisms that stimulate the formation of the GTP-bound state of the newly translated TGase protein are currently unknown. Expanding upon our finding that RA requires active PI3K to induce TGase expression, we asked whether PI3K expression following exposure to RA might also affect TGase GTP binding activity independent of the effects on TGase expression. To examine this possibility, we took advantage of previous work that showed that expression of exogenous TGase in cells yielded a TGase species, which was able to bind GTP even without the addition of the stimulatory factor RA (7). Consistent with this finding, NIH3T3 cells transiently expressing a TGase construct incorporated [α-32P]GTP (Fig. 4A, inset) in a manner similar to the TGase GTP binding activity observed when cells were treated with RA for several days. When duplicate plates of cells expressing exogenous TGase were exposed to LY294002 for 2 days, the ability of the TGase to incorporate [α-32P]GTP was significantly reduced (Fig. 4A, inset and graph); the average inhibition in several experiments was ~60%. However, even in the presence of excess concentrations of LY294002, the GTP binding activity of the TGase was not completely eliminated (data not shown), suggesting that a PI3K-independent pathway can contribute to the activation of TGase GTP binding activity. To ensure that the reduction in the GTP binding activity of the exogenous TGase by the chemical inhibitor was a result of down-regulating PI3K activity rather than the result of a nonspecific inhibitory effect on the TGase molecule, we tested whether the GTP binding activity of recombinant purified TGase was altered in the presence of LY294002. Fig. 4B showed that incubating 25–50 ng of recombinant TGase with increasing concentrations of LY294002 (up to 400 μM) did not change the ability of the TGase to bind GTP as read out by photoaffinity-labeling with [α-32P]GTP. How the signaling capability of PI3K promotes the binding of GTP to the TGase in cells is not understood and is a current focus of our research. One intriguing possibility is that PI3K mediates the expression and/or activation of a guanine nucleotide exchange factor for TGase. Once up-regulated, the

exchange factor could promote the binding of GTP to the TGase in a manner analogous to the function of guanine nucleotide exchange factors on members of the Ras family of small G-proteins (28). Another possibility may involve the ability of PI3K signaling to limit the effectiveness of a negative regulator(s) of TGase activity that is known to exist in cells (7, 21).
The RA-induced stimulation of PI3K activity could produce a signaling event that would somehow disrupt the interaction of the TGase with the inhibitory complex, making the TGase accessible for binding GTP.

Having established a link between PI3K and TGase activation, we then examined whether cell fate responses induced by RA would be affected if PI3K activity was inhibited. Under the stress of serum starvation, NIH3T3 cells exposed to RA for nearly 2 days displayed a low rate of apoptosis, whereas the treatment with the related retinoid, HPR, induced a potent cell death response (Fig. 5). Because RA but not HPR was shown to enhance TGase expression and activation, it was proposed that this distinct signaling feature may be important for the anti-apoptotic effects of RA (7). Consistent with this idea, RA-stimulated cells pretreated with MDC, a competitive inhibitor of the TGase-catalyzed transamination, displayed an ~35% decrease in cell viability (Fig. 5), indicating that the exposure of cells to RA could be sensitized to apoptosis by simply limiting the function of the TGase. Because PI3K activity is required for the RA-induced up-regulation of TGase activity and TGase activity appears to be critical for the survival effect of RA, we examined whether the treatment of RA-stimulated cells with LY294002 gave rise to an increase in cell death. This in fact was the case as the addition of RA to cells treated with LY294002 resulted in a significant increase in the number of apoptotic cells. Still, the extent of apoptosis was always less than that of the accompanying HPR treatment, most probably because typically some amount of PI3K activity and corresponding TGase expression and activation persists even in the presence of the inhibitor. However, TGase expression and activation can be completely eliminated when combining LY294002 with MDC, thus yielding a level of apoptosis that was nearly equivalent to that induced by HPR. Taken together, these data strongly suggest that PI3K-mediated TGase activation is a key element of the survival response afforded by RA.

TGase activation has largely been associated with programmed cell death (5, 6, 29); however, more recently, TGase function has been shown either not to be directly linked to the apoptotic process (8, 30) or to have an anti-apoptotic role in cells (7). The fact that we have now demonstrated that RA-mediated TGase expression and activation require the activation of the well established survival factor PI3K further implicates TGase in a survival role. At present, we do not know how PI3K signaling influences the expression of the TGase, although presumably it will involve the activation of one or more known PI3K effectors such as ribosomal S6 kinase or AKT. AKT is an especially interesting possibility, because it has been implicated in promoting the expression of survival genes by activating the cAMP-responsive element-binding protein and the nuclear Xb transcription factors (31).

Thus, in summary, we have shown for the first time that RA activates the signaling molecule, PI3K, and that this activation is essential for the ability of RA to induce the expression and subsequent activation of the TGase. How both PI3K and ERK become activated following RA stimulation is unclear and warrants further investigation. The RA receptors are nuclear receptors that upon binding RA become active transcription factors, which modulate the expression of RA-responsive genes (13, 14). Although it is generally believed that RA-induced cellular responses occur via the up-regulation of gene expression, our data imply that RA is capable of directly activating similar intracellular signaling cascades as those activated by growth factors. In support of this idea, RA treatment has previously been reported to increase the activation of several intracellular signaling proteins including focal adhesion kinase (32), ERK (24), and PI3K (25). We are beginning to evaluate whether RA can activate additional signaling cascades that contribute to the up-regulation of TGase expression and/or its activation.

Acknowledgment—We acknowledge the expert secretarial assistance of Cindy Westmiller.

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