Transcript Inhibition of Protein Synthesis Induces the Immediate Early Gene VL30: Alternative Mechanism for Thapsigargin-induced Gene Expression

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Abstract
Induction of gene expression in response to calcium ionophores or thapsigargin, which inhibits the calcium-ATPase responsible for sequestering intracellular calcium, has frequently been attributed to direct stimulatory events subsequent to the elevation of intracellular free calcium. VL30 is a murine gene that is transcriptionally induced in response to a large array of mitogenic and transforming stimuli. We have shown previously that an enhancer element within the VL30 promoter region is dependent upon cotreatment with thapsigargin or calcium ionophore for a full-scale induction of gene expression. In this report, we demonstrate that both thapsigargin and calcium ionophores induce a transient inhibition of protein synthesis in Rat-1 cells transfected with a VL30 enhancer-driven reporter construct. Recovery of protein synthesis is facilitated by cotreatment with epidermal growth factor or phorbol esters. Furthermore, treatment with cycloheximide or DTT, which inhibit protein synthesis without altering intracellular calcium levels, can substitute for thapsigargin or ionophores in stimulating VL30 gene expression. These results suggest that the stimulatory effects of thapsigargin and calcium ionophores on VL30 expression may be mediated, at least in part, by the ability of these agents to initiate stress responses associated with the inhibition of protein synthesis.

Introduction
Events leading to an increase in free cytosolic calcium have been associated with increased expression of a number of eukaryotic genes, including the immediate early genes c-fos, c-jun and c-myc (1–3) as well as other genes such as prolactin (4) and the glucose-regulated proteins GRP78 and GRP94 (5). The pathways linking changes in intracellular calcium levels to changes in gene expression are complex, involving multiple, calcium-sensitive kinases as well as distinct, calcium-responsive enhancer sequences. This complexity has made it difficult to identify the specific mechanisms involved in the calcium-sensitive induction of a given gene.

Experimental manipulation of intracellular calcium levels by TG and calcium ionophores is complicated by many factors. Although calcium ionophores like ionomycin and A23187 facilitate the entry of extracellular calcium into cells, these agents also interact with the membranes of the endoplasmic reticulum to facilitate loss of calcium from intracellular stores. TG acts by inhibiting the Ca2+-ATPase responsible for sequestering cytosolic calcium within the endoplasmic reticulum, so that free cytosolic calcium levels are increased while the concentration of calcium within the endoplasmic reticulum is actually decreased (6–9). Decreased calcium concentration within the endoplasmic reticulum subsequent to TG or ionophore treatment has been demonstrated to inhibit protein synthesis at the point of translation initiation (9–12). Thus, it is possible that the increase in gene expression observed in response to treatment with calcium ionophores or TG may reflect either stimulatory effects of elevated cytosolic calcium concentrations or the activation of stress-related signaling pathways sensitive to changes in protein synthesis.

In this report, we have used the proliferation- and transformation-associatedVL30 enhancer element to investigate the mechanisms by which TG and ionophores stimulate calcium-dependent gene expression. VL30 genes in the mouse genome consist of a family of retroviral elements that resemble immediate early genes in their responsiveness to growth factors and activated signaling pathways (13–15). The VL30 long terminal repeat contains a triply repeated sequence that is capable of serving as a classical enhancer element in a variety of mammalian cell types (16). Although the VL30 enhancer element mediates a modest increase in gene expression in response to EGF, TPA, or B2cAMP when each agent is used singly, simultaneous exposure to these agonists and either TG or calcium ionophore produces an extremely robust increase in gene expression (17, 18). Molecular analyses have indicated that each of these signaling pathways (e.g., tyrosine kinase receptors, protein kinase C, protein kinase A, and calcium) converge upon the same 20-nucleotide sequence (5’-TGATCCCTAGTTAGTGTTAGG-3’), capable of specific protein binding in both DNase foot printing and electrophoretic mobility shift assays (18). The synergistic interactions between calcium-modulating agents and other agonists provide a sensitive assay system for determining the effectiveness of elevated calcium versus calcium depletion in modulating gene expression. The results described in this report indicate that inhibition of protein synthesis and the subsequent activation of stress-related signaling pathways play a major role in increasing VL30 gene expression in response to TG or ionophore treatment.

Results
Induction of VL3R-TK-CAT Expression by TG, Ionomycin, and DTT. Exposure of cells in culture to low concentrations of DTT has been demonstrated to inhibit protein synthesis in a calcium-independent fashion (19). Thus, if the ability of TG to induce gene expression from the VL30 triple repeat is related to inhibition of protein synthesis, treatment with

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3 The abbreviations used are: TG, thapsigargin; EGF, epidermal growth factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; B2cAMP, dibutyryl adenosine 3’-5’-cyclic monophosphate; CAT, chloramphenicol acetyltransferase.
D TT should produce similar changes in gene expression. This hypothesis was tested by exposing VL3R-TK-CAT cells to either 2 µg TG, 1 µg ionomycin, or 0.8 mM DTT in the presence or absence of 10 ng/ml EGF, 100 ng/ml TPA, or 0.6 mM Bt2cAMP. When added singly, each of the aforementioned agents elicited only a small induction of CAT activity (Fig. 1). When each of the agonists (EGF, TPA, or Bt2cAMP) was combined with any of the three agents tested (TG, ionomycin, or DTT), a robust induction of CAT activity was observed. An exception was the lack of induction of CAT activity by the combination of DTT and EGF. Further tests demonstrated that the addition of DTT to the culture medium resulted in substantial inactivation of EGF, presumably as a result of reduction of disulfide groups known to be essential for the biological activity of EGF (results not shown).

Inhibition of Protein Synthesis by TG and Ionomycin.

The ability of DTT to synergize with TPA or Bt2cAMP suggested that the inductive response to these agents may reflect their ability to inhibit protein synthesis. This interpretation is further supported by the demonstrated ability of cycloheximide to produce a superinduction of genomic VL30 mRNA (14). However, in order to observe increased CAT expression, protein translation must recover to permit the translation of CAT mRNA into protein. Inhibition of protein synthesis in Rat-1 cells by TG, ionomycin, or DTT would, therefore, need to be transient in order to produce the observed increase in CAT enzyme activity.

To determine the temporal effects of TG on protein synthesis in this experimental system, we exposed VL3R-TK-CAT cells to TG in the presence or absence of EGF, TPA, or Bt2cAMP (Fig. 2A). Incorporation of [3H]leucine into acid-precipitable material was then measured during labeling intervals of 15 min at varying times following the addition of agonists to cells. In other experiments, we confirmed that the incorporation of acid-soluble [3H]leucine into cells was linear within the pulsing interval (data not shown). Results of the experiment shown in Fig. 2A demonstrate that 30 min after the addition of TG, the incorporation of [3H]leucine was reduced to approximately 10% of control values. Similar values were observed when TG was co-added with either Bt2cAMP, EGF, or TPA. Two h after the addition of TG alone, the incorporation of [3H]leucine into cells had increased to approximately 16% of control values. In contrast, the addition of either EGF, TPA, or Bt2cAMP in the presence of TG stimulated recovery of [3H]leucine incorporation to approximately 35–40% of control values within 2 h. Fig. 2B shows that cells exposed to ionomycin and DTT similarly demonstrated approximately 85% inhibition of incorporation of [3H]leucine within 30 min. Recovery of [3H]leucine incorporation returned nearly to control values by 2 h for cells exposed to DTT. Cells exposed to ionomycin...
behaved in a similar manner to those treated with TG; there was little recovery of \(^{1}H\)leucine incorporation at 2 h in cells exposed to ionomycin alone, but cells exposed to ionomycin plus either EGF or Bt2cAMP recovered to approximately 40% of control levels.

**Effect of TG and Ionomycin on Translation Initiation.** To determine whether TG, ionomycin, or DTT was inhibiting protein synthesis by acting at the level of translation initiation, polysome profiles were obtained from VL3R-TK-CAT cells that had been treated with these agents for 30 min. Gradient profiles from serum-deprived control cells demonstrated a rapidly sedimenting region of high absorbance, characteristic of polysomes (Fig. 3A, brackets) and a peak of 80S monosomes. Regions of the gradient shown in Fig. 3A were examined by Northern blot analysis for mRNA corresponding to cyclophilin, a constitutively expressed mRNA (20, 21). The mRNA was contained exclusively in polysome fractions (data not shown). Following exposure of cells to either TG, ionomycin, or DTT, polysome accumulation was halted, as demonstrated by the marked decrease in absorbance of gradient fractions corresponding to the polysome peak. Additionally, there occurred a substantial increase in absorbance of fractions corresponding to the 80S monosome peak and in other regions near the top of the gradient. Examination of cyclophilin expression by Northern blot analysis in gradient fractions from cells exposed to TG for 30 min revealed the major concentration of cyclophilin mRNA in fractions corresponding to the 80S monosome peak (data not shown). These data demonstrated that exposure to TG, ionomycin, and DTT produced a similar conversion of polysomes to monosomes, consistent with an induced decrease in the rate of translation initiation. Gradient profiles of untreated control cells and cells treated with cycloheximide were identical (data not shown), consistent with the known ability of cycloheximide to inhibit translation at the level of elongation rather than initiation (22).

Recovery of polysome formation was examined in cells exposed to TG for an extended time in the presence or absence of EGF (Fig. 3B). When compared with cells treated for 30 min with TG alone, cells examined 4 h after the addition of TG showed a partial return of polysomes to the gradient profiles and a concomitant decrease in the 80S and other peaks in upper gradient fractions. Cells treated with EGF plus TG for 4 h showed a more pronounced recovery of absorbance in the region of polysomes and a greater decrease in the 80S monosome peak; these profiles were indistinguishable from the gradient profile of untreated control cells in Fig. 3A. Inhibition of RNA synthesis
Inhibition of Protein Synthesis Induces VL30

by actinomycin D prevented the restoration of polysome formation in the presence of EGF plus TG (Fig. 3B).

**Effect of Transient Inhibition of Protein Synthesis on VL3R-TK-CAT Expression.** The data presented above have demonstrated that EGF, TPA, and Bt2cAMP promote the recovery of protein synthesis following inhibition induced by TG or ionomycin treatment. We reasoned that the large increase in CAT enzyme activity that occurred 4 h after cotreatment with these agents may reflect the accumulation of VL3R-TK-CAT mRNA in the presence of protein synthesis inhibitors; this mRNA is then translated into CAT protein following restoration of protein synthesis. To test this model, we determined whether transient exposure of cells to chemical inhibitors of protein synthesis such as cycloheximide could mimic the effects of TG on induction of VL3R-TK-CAT activity. VL3R-TK-CAT cells were preincubated with 25 μg/ml cycloheximide for varying times. Cells were then rinsed free of cycloheximide and postincubated for 4 h in serum-free medium prior to harvest. Results of this experiment demonstrated that preincubation in either Bt2cAMP or cycloheximide alone failed to induce substantial CAT activity during the postincubation period (Fig. 4). However, exposure to the combination of Bt2cAMP and cycloheximide during the preincubation period produced a robust induction of CAT activity, and a nearly linear relationship was observed between the duration of exposure to Bt2cAMP plus cycloheximide and the induction of CAT activity. This experiment suggested that a decreased rate of protein synthesis in the presence of Bt2cAMP produced a continuing transduction of cellular signals over the entire period of cell exposure. In other experiments, we demonstrated that preincubation of cells in cycloheximide plus either TPA or EGF produced similar results (data not shown). We tested a number of other protein synthesis inhibitors, all of which proved capable of synergizing with Bt2cAMP in the induction of VL3R-TK-CAT activity (Table 1). Taken together, these data demonstrated that a transient interruption of protein synthesis in the presence of agonist was capable of inducing CAT enzyme activity from the VL3R-TK-CAT reporter construct, as measured following restoration of protein synthesis.

**Induction of VL3R-TK-CAT mRNA by TG.** Cellular expression of CAT enzyme activity driven by reporter gene constructs may not bear a direct relationship to the induced synthesis of CAT mRNA, especially when translation initiation rates are impaired by compounds such as TG, ionomycin, and DTT. To measure changes in VL3R-TK-CAT mRNA expression directly, we performed Northern blot analysis of cellular RNA using a radiolabeled fragment of the CAT DNA sequence as the hybridizing probe (Fig. 5). CAT RNA was detected as doublet bands on Northern blots. Exposure of VL3R-TK-CAT cells to EGF produced a small increase in CAT mRNA at 30 min, which was followed by a rapid decline. Exposure of cells to TG induced higher levels of CAT mRNA than did EGF alone; maximal levels were attained by 30 min and declined slowly over the next 2–4 h. Combined treatment of cells with EGF and TG induced CAT mRNA to levels much higher than observed with either agent added singly and declined with the same kinetics as cells treated with TG alone. The Northern blot shown in Fig. 5 demonstrates the existence of two bands of CAT mRNA that respond to induction similarly but that appear to be degraded with different kinetics. Parallel experiments substituting either TPA or Bt2cAMP for EGF also demonstrated a synergistic induction of CAT mRNA when added in the presence of TG (data not shown).

The increase in CAT mRNA accumulation observed by Northern analysis could result from an increase in the half-life of CAT mRNA following inhibition of protein synthesis. We tested this possibility directly by measuring the half-life of CAT mRNA in the presence or absence of cycloheximide. After a two-h induction of CAT mRNA with EGF + cycloheximide, cells were rinsed free of agonist-containing media and incubated with 25 μg/ml actinomycin D in the presence or absence of fresh cycloheximide (Fig. 6). The half-life of CAT mRNA in control cells was 5.1 ± 1.3 h, while cycloheximide treatment actually decreased the half-life to 3.8 ± 0.7 h; this difference is not statistically

**Table 1 Effect of protein synthesis inhibitors**

<table>
<thead>
<tr>
<th>Additive(s)</th>
<th>CAT activity (Fold induction)</th>
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<tbody>
<tr>
<td>Bt2cAMP</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td>CHX (25 μg/ml)</td>
<td>37 ± 4.0</td>
</tr>
<tr>
<td>Bt2cAMP + CHX (25 μg/ml)</td>
<td>334 ± 17</td>
</tr>
<tr>
<td>Puromycin (50 μg/ml)</td>
<td>28 ± 1.2</td>
</tr>
<tr>
<td>Bt2cAMP + puromycin (50 μg/ml)</td>
<td>260 ± 6.7</td>
</tr>
<tr>
<td>Anisomycin (10 μg/ml)</td>
<td>25 ± 3.4</td>
</tr>
<tr>
<td>Bt2cAMP + anisomycin (10 μg/ml)</td>
<td>288 ± 7.7</td>
</tr>
<tr>
<td>Pautamycin (5 ng/ml)</td>
<td>10 ± 1.6</td>
</tr>
<tr>
<td>Bt2cAMP + pautamycin (5 ng/ml)</td>
<td>203 ± 39</td>
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significant. This result indicates that the observed increase in CAT mRNA accumulation was not due to enhanced stability in the absence of protein synthesis.

**Discussion**

Induction of mRNA expression from the VL30 triple repeat element is a complex process that appears to require interactions between multiple signal transduction pathways in order to obtain a significant increase in expression. In previous work from this laboratory, we have shown that agents that elevate intracellular calcium levels, either by stimulating release of intracellular calcium stores (TG or IP$_3$-elevating agents) or by stimulating calcium influx (ionomycin or A23187) produce only modest increases in CAT expression from the VL30 triple repeat-CAT expression vector VL3R-TK-CAT. Similarly, agents such as EGF, TPA, or Bt$_2$cAMP that activate specific protein kinases are also relatively inefficient inducing agents when used singly. However, combinations of calcium-modulating reagents and protein kinase activators produce robust increases in VL3R-TK-CAT expression, sometimes approaching 40- to 50-fold over basal levels.

In this report, we have attempted to identify a mechanism contributing to the synergistic interaction between calcium-modulating agents and other agonists in the induction of VL3R-TK-CAT expression. We have presented data demonstrating that exposing Rat-1 cells to TG or ionomycin produced a transient inhibition of protein synthesis, as demonstrated by both an inhibition of incorporation of $[^{3}H]$leucine into acid-insoluble material and the conversion of polysomes to monosomes and subribosomal fragments. This is in agreement with the studies of Wong et al. (9), Chin et al. (22), and Brostrom et al. (23), who concluded that TG and ionophores inhibit protein synthesis at the level of translation initiation.

The observation of reduced protein synthesis in response to TG or ionophore treatment of Rat-1 cells raises the possibility that these agents may be inducing VL3R-TK-CAT expression via a "stress pathway" activated when protein synthesis is inhibited. This interpretation is supported by the
demonstration that cycloheximide and DTT, agents that inhibit protein synthesis without modifying intracellular calcium levels (19, 24), are also capable of interacting synergistically with EGF, TPA, or Bt2cAMP to stimulate VL3R-TK-CAT expression. In our experiments, the inhibition of protein synthesis was gradually released over a period of several hours in the absence of other treatments; however, cotreatment with EGF or TPA greatly hastened the rate at which protein synthesis returned to normal levels. These results raise the possibility that the increase in VL3R-TK-CAT enzyme activity observed in response to combinations of TG and EGF or TPA represents a direct stimulatory effect of protein synthesis inhibition on the induction of CAT mRNA driven by the VL30 enhancer, coupled with an accelerated recovery of CAT protein synthesis in the presence of EGF or TPA. The transient inhibition of protein synthesis is sufficient for the synergistic increase in CAT mRNA induction observed when agonists such as EGF, TPA, or Bt2cAMP are used in conjunction with TG. The increase in VL3R-TK-CAT mRNA expression observed when protein synthesis is inhibited could not be explained by an increased half-life, because cycloheximide treatment actually decreased CAT mRNA half-life. Our ability to observe an increase in CAT enzyme activity was dependent upon the recovery of protein synthesis, and the ability of EGF or TPA to enhance the recovery process may have also contributed to the increase in CAT activity.

This interpretation of our results is consistent with a substantial accumulation of evidence linking calcium homeostasis to the regulation of protein synthesis and gene expression. Elegant biochemical studies from the laboratories of Brostrom et al. (10, 11) and Chin et al. (22) have demonstrated that the initiation of protein translation is dependent upon the presence of adequate calcium stores within the endoplasmic reticulum. Furthermore, exposure of cells to calcium ionophores or TG results in a net depletion of sequestered calcium and a subsequent inhibition of protein synthesis (9, 11). These investigators have also observed a stimulatory effect of phorbol esters and cAMP on protein synthesis, and they have linked this adaptation response to the transcriptional induction of GRP78 (23, 25).

In summary, the data presented in this report indicate that VL30 expression is in many aspects compatible with that of stress-related genes. Although we have been unable to separate the elevation of cytosolic calcium in response to TG or ionomycin from the inhibition of protein synthesis induced by these agents, we have shown that inhibition of protein synthesis alone is sufficient for the increased induction of VL3R-TK-CAT. A large number of reports have used ionophores such as A23187 or ionomycin as evidence for the involvement of elevated intracellular calcium in the induction of gene expression (1, 4, 26–31). Several reports have defined "calcium response elements" solely on the basis of inducibility by calcium ionophores (1, 27, 28). The transient decrease in translation initiation induced by these ionophores and other agents following loss of calcium from the endoplasmic reticulum may provide a novel signaling mechanism for the induction of gene expression. Elucidating the mechanisms that control these protein synthesis-dependent pathways may reveal how VL30 and other immediate early genes are regulated by physiological and nonphysiological agents that mobilize calcium from cellular stores.

Materials and Methods

Cell Culture. Rat-1 cells (32) and stably transformed Rat-1 derivatives were maintained in DMEM plus 10% defined calf serum (HyClone) in a 37°C–95% air/5% CO₂ incubator. Cultures were supplemented with 10 μg/ml Gentamicin and amphotericin B (Fungizone; Sigma Chemical Co.). Stably transfected clonal cell lines were selected and cultured in the presence of 750 μg/ml G418 (GIBCO).

Plasmids and Transfected Cell Lines. Construction of the plasmid pcTK3R has been described previously (17). In pcTK3R, CAT expression is driven by a triple repeat of the VL30 35-bp enhancer element in the context of the Herpes simplex thymidine kinase promoter.

Rat-1 cells were stably transfected with the plasmids pcTK3R and pk0-neo to produce the representative stable cell line TK3R-3cat, which was selected because its responsiveness to EGF and TPA resembled that of Rat-1 cells transiently transfected with pcTK3R (17). We have renamed this cell line VL3R-TK-CAT to more accurately reflect the genetic element driving CAT expression.

Probes for Northern hybridization analyses were obtained by hexamer labeling of restriction fragments released from the appropriate plasmid. For analysis of CAT mRNA, CAT coding sequence was excised from pCATbasic (Promega) using XbaI and EcoRI.

Measurement of CAT Activity. Cellular proteins were extracted essentially as described in Gorman et al. (33), and the cell extracts were incubated for 10 min at 70°C to inhibit cellular acetylases. CAT activity was measured by the two-phase CAT assay of Neumann (34); only data from the linear portion of the reaction curve were used.

Northern Hybridization Analysis of RNA. At appropriate intervals after stimulation, cells were harvested, and total cellular RNA was extracted as described previously (14). The RNA was size fractionated by electrophoresis in 1.2% agarose/formaldehyde gels and transferred to nylon membranes (Nytran; Schleicher & Schuell, Keene, NH). Hybridization probes were labeled with [32P]dCTP by random hexamer extension using a commercial kit (NEN Research Products); specific activities of 0.5–1 × 10⁶ dpm/μg were obtained routinely. Autoradiography was conducted at −70°C with intensifying screens for the times indicated.

Sucrose Gradient Analysis of Polysomes. Polysome size and abundance was determined essentially as described by Chin et al. (22). Following experimental treatment, cells were suspended in 150 mm NaCl-10 mm Na₂HPO₄ (pH 7.4) and centrifuged at 1000 × g. The cell pellet was lysed in 1.2 ml of 50 mm Tris (pH 7.4), 26 mm KCl, 5 mm MgCl₂, 1% Triton X-100, 1% sodium deoxycholate, 1 mg/ml heparin, and 10 μg/ml cycloheximide. Cell lysates were centrifuged at 10,000 × g for 10 min, and 200 μl of the resulting supernatant were applied to an 11-m1 17–51% linear sucrose gradient in 50 mm Tris (pH 7.4), 25 mm KCl, and 5 mm MgCl₂. The gradients were centrifuged at 270,000 × g for 2 h in an SW 28 rotor. Gradients were pumped through a UV-1 optical monitor containing a 254-nm filter (Pharmacia) by upward displacement at 2.2 ml/min. Each gradient contained cell extract derived from three 10-cm culture dishes (approximately 3 × 10⁶ cells).

Reagents. TG (LC Services, Woburn, MA) was dissolved in DMSO and stored at −20°C prior to use. EGF, TPA, ionomycin, Bt2cAMP, cycloheximide, anisomycin, and DTT were obtained from Sigma Chemical Co. (St. Louis, MO) and were dissolved in the appropriate vehicle (DMSO).
for TPA, ionomycin, and TG; dDTP for B2, cAMP). Restriction endonucleases and other DNA-modifying enzymes were purchased from Bethesda Research Labs (Gaithersburg, MD).

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