Heterologous Expression of the Transcriptional Regulator Escargot Inhibits Megakaryocytic Endomitosis*

Received for publication, June 28, 2001, and in revised form, July 31, 2001
Published, JBC Papers in Press, August 9, 2001, DOI 10.1074/jbc.M106006200

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Certain cell types escape the strict mechanisms imposed on the majority of somatic cells to ensure the faithful inheritance of parental DNA content. This is the case in many embryonic tissues and certain adult cells such as mammalian hepatocytes and megakaryocytes. Megakaryocytic endomitosis is characterized by repeated S phases followed by abortive mitoses, resulting in mononucleated polyploid cells. Several cell cycle regulators have been proposed to play an active role in megakaryocytic polyploidization; however, little is known about upstream factors that could control endomitosis. Here we show that ectopic expression of the transcriptional repressor escargot interferes with the establishment of megakaryocytic endomitosis. Phorbol ester-induced polyploidization was inhibited in stably transfected megakaryoblastic HEL cells constitutively expressing escargot. Analysis of the expression and activity of different cell cycle factors revealed that Escargot affects the G1/S transition by influencing Cdk2 activity and cyclin A transcription. Nuclear proteins that specifically bind the Escargot-binding element were detected in endomitotic and non-endomitotic megakaryoblastic cells, but down-regulation occurred only during differentiation of cells that become polyploid. As Escargot was originally implicated in ploidy maintenance of Drosophila embryonic and larval cells, our results suggest that polyploidization in megakaryocytes might respond to mechanisms conserved from early development to adult cells that need to escape normal control of the diploid state.

In most somatic cells, the ploidy of progeny is maintained by tightly controlling the proper alternation of DNA replication and mitosis. However, polyploidization occurs in different cell types from plants to vertebrates as part of their physiological differentiation programs. For instance, most embryonic cells that do not give rise to adult structures are highly polyploid, probably as a means to potentiate the high metabolic rate needed to sustain the developing embryo (1). In adult tissues, a few highly differentiated and mature cells also appear to require an increase in DNA content, as is the case for mammalian hepatocytes and the platelet precursor cell, the megakaryocyte. In the final stages of maturation, megakaryoblasts stop proliferating and undergo from three to five truncated cell cycles, resulting in polyploid, fully mature megakaryocytes.

To increase their DNA content to levels up to 128N, megakaryocytes repeatedly enter S phase after escaping mitosis without completing anaphase in a process traditionally called endomitosis (2, 3). In vitro and in vivo experimental approaches have shown that both G1/S and G2/M cell cycle transitions have to be regulated for megakaryocytes to achieve polyploidization (4–12). Specifically, both cyclin D3-Cdk2 and cyclin E-Cdk2 complexes have been proposed as the most likely candidates to drive megakaryocytic polyploidization (4, 11, 12). However, little is known about the mechanisms upstream of the cell cycle machinery that ultimately control the proper establishment of endomitotic cycles in differentiating megakaryoblasts.

In embryonic systems, the entrance into re-replication cycles seems to be inhibited by proteins belonging to the Snail family of transcriptional repressors (for recent reviews, see Refs. 13 and 14). All Snail proteins contain four to six highly conserved zinc fingers at the C terminus of the protein through which they bind DNA. In vitro binding site selection experiments using pools of random sequences have revealed that the consensus binding element corresponds to an E2 box that is recognized by a variety of basic helix-loop-helix transcriptional activators. It has been proposed that the Snail proteins may compete directly with basic helix-loop-helix proteins for the same binding sequences, although this has been observed only in a limited number of cases (19, 20). Genetic studies in Drosophila, Xenopus, Caenorhabditis elegans, and mouse have shown that Snail proteins participate in a variety of processes that include mesoderm determination, neurogenesis, and apoptosis (13, 14). More recently, Snail proteins have been implicated in the invasiveness of cancer cells (15, 16) and in the development of B lymphomas (17), revealing that they might have important roles in adult tissues.

Escargot was first described in relation to the control of Drosophila imaginal cell development (18). Further analysis revealed that Escargot is involved in the maintenance of diploidy in embryonic and larval cells (19). Thus, ectopic expression of escargot inhibits polyploidization in salivary glands, whereas its ablation prevents the regular progression through mitotic cycles in the imaginal tissues and, as a result, causes abdominal histoblasts to undergo endocycles and become polyploid (18, 19). Interestingly, Escargot inhibits polyploidization of murine trophoblast giant cells as efficiently as its murine homolog, Snail (20). In addition, the endogenous expression pattern of both escargot and snail further suggests an inhibitory activity on Drosophila and mouse embryonic endo-
cycles, respectively, as both proteins are down-regulated in those cells that become polyploid (18, 20).

Here we have asked whether a similar role could be played by proteins related to Escargot in megakaryocytic endomitosis. The megakaryoblastic cell line HEL normally responds to the phorbol ester TPA by undergoing terminal differentiation including polyploidization. We have isolated stable clones of HEL cells constitutively expressing escargot and monitored their response to TPA in terms of differentiation marker expression, extent of polyploidization, and expression and activity of different cell cycle regulators. In addition, we have explored whether the presence of endogenous Escargot-like proteins capable of specifically binding to an Escargot-binding element (EBE) correlates with the ability of megakaryoblastic cells to become polyploid. Our results show that escargot expression interferes with the regulation of the G1/S transition of endomitotic cycles and thereby with megakaryocytic polyploidization.

They also suggest that down-regulation of proteins that specifically interact with the EBE could be required for megakaryoblastic cells to become polyploid.

MATERIALS AND METHODS

Culture Cells — Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (BioWhittaker), 2 mM L-glutamine, and 80 μg/ml gentamicin. Cells were maintained at 37 °C under 5% CO2 and 95% air in a humidified incubator. In all experiments, exponentially growing cells were subcultured into Nunc 96-, 24-, or 6-well plates with or without appropriate treatment. The number of viable cells was determined by trypan blue exclusion in a hemocytometer.

 Constructs and Oligonucleotides — Full-length escargot DNA was obtained from Dr. Mary Whiteley. Expression vector pcDNA3-ESG was generated by subcloning an XhoI fragment containing the ESG open reading frame into the pcDNA3 polylinking site (Invitrogen). The construction of the cyclin A promoter has been described elsewhere (11).

Transfection and Isolation of escargot-expressing HEL Cells — HEL cells (American Type Culture Collection) were transected by electroporation at 125 microfarads/300 V in a 0.4-cm cuvette. 5 × 106 cells were transfected with 5 μg of linearized pcDNA3 or pcDNA3-ESG plasmids. After 24 h, cells were washed with phosphate-buffered saline and respursed in fresh medium supplemented with 50% G418 (Gibco-BRL, Life Technologies, Inc.). Cells were subcultured in 96-well plates at 1 × 106 cells/ml. Single cell clones were isolated by limiting dilution of the G418-resistant cells.

For transient transfection experiments, cell lines were transfected by electroporation. 2 × 106 cells were transfected with 2.5 μg of plasmid DNA by electroporation. For TPA treatments, electroporated cells were divided into two aliquots and allowed to recover for 12 h. One aliquot was then treated with TPA. 48 h after electroporation, the cells were harvested, and cell extracts for assessing luciferase activity were made by three cycles of freeze-thaw lysis.

Phenotypic Characterization of Cells — Morphological Wright stain was performed on cytospun cells. Cell-surface immunofluorescent staining was performed on 106 cells/ml with antibodies against glycoprotein IIIa/CD61 or glycophorin A conjugated to FITC (Becton Dickinson) as previously described (11). DNA content was determined by staining with 50 μg/ml propidium iodide. Cell cycle analysis was performed using a FACScan analyzer using CellQuest software (Becton Dickinson).

RESULTS

Constitutive Expression of escargot Does Not Affect the Growth or Morphology of Megakaryoblastic HEL Cells — Endomitotic megakaryoblastic HEL cells were transfected with full-length Esg cDNA under the control of a cytomegalovirus promoter. 10 clones were selected in the presence of G418 and analyzed for the presence of functional ESG protein. Nuclear extracts of transfectants were analyzed by electrophoretic mobility shift assays using a labeled double-stranded oligonucleotide containing the consensus binding site for ESG (ACAG-TGT, hereinafter referred to as the EBE). As shown in Fig. 1a, extracts from four of the clones transfected with the ESG-expressing vector gave a pattern of shifted proteins that was not present in a G418-resistant clone of HEL cells transfected with an empty vector. The latter was subsequently used as control for HEL (referred to as HEL-26E cells). A similar pattern of protein-EBE complexes was observed in COS cells transiently transfected with the same ESG-expressing construct (Fig. 1c).

The specificity of binding to the EBE of proteins derived from the HEL/ESG clones was assessed by competing with a 100-fold excess of the EBE oligonucleotide, a mutant version of the EBE that does not bind to ESG (ESGmut), or an oligonucleotide containing an unrelated sequence. This revealed that the two predominant complexes of higher mobility represented specific
one of the proteins. Plasmids and selected in G418 under the conditions described under HEL cells were transfected with empty pcDNA3 or pcDNA3-ESG the TPA-driven up-regulation of megakaryocytic markers such as glycoprotein IIIa (CD61) and down-regulation of the erythroid marker glycophorin A remained unchanged in the ESG-expressing cells compared with HELc cells (Fig. 2b). The morphology of exponentially growing HELc and ESG-expressing clones was very similar, with a blast morphology typical of erythroleukemic cells, whereas ESG-expressing HEL cells showed no cytoplasmic or nuclear maturation and the presence of large vacuoles in a high proportion of cells. Therefore, it appeared that ESG was specifically interfering with the completion of the TPA-driven megakaryocytic differentiation program.

**Escargot Interferes with Phorbol Ester-induced Polyploidization—**Next we analyzed the nuclear DNA content of TPA-treated cells to determine whether the key feature of megakaryocytic maturation, i.e. the degree of polyploidization, was also affected in the presence of ESG. Control and ESG-expressing HELc clones were seeded at 1.5 × 10^5 cells/ml in the presence or absence of TPA and stained with propidium iodide (48 and 96 h after treatment). More than 50% of the HELc cells showed a DNA content of 4N or higher (10 and 1% of cells being 8N and 16N, respectively) 48 h after treatment, whereas HA1 and HD12 cells failed to become polyploid even after 96 h in the presence of TPA (Fig. 3). Instead, the propidium iodide staining pattern indicated that ESG-expressing HEL cells remained arrested in G1 and G2. This behavior appeared to be reminiscent of megakaryoblastic K562 cells, which respond to TPA in terms of platelet antigen up-regulation, but do not become polyploid (Fig. 3). As ESG DNA-binding properties were not affected by the TPA treatment (data not shown), we conclude that Escargot inhibits the establishment of endomitotic cycles in megakaryoblastic cells.

**Escargot-mediated Inhibition of Megakaryocytic Polyploidization Occurs through Alteration of the Endomitotic G1/S Transition—**Establishment of endomitotic cycles in HEL cells requires...
active cyclin E-Cdk2 complexes, which in turn maintain cyclin A expression (11). We therefore analyzed the G1/S machinery in HELc cells and ESG-expressing clones before and after treatment with TPA. As shown in Fig. 4a, TPA treatment of HA1 and HD12 cells resulted in a decrease in the levels of cyclins E and A and Cdk2 activity, whereas p27kip1 inhibitor levels remained unchanged or even increased. This was in sharp contrast with the pattern of expression of these proteins in HELc cells, which showed the expected maintenance of cyclin and p27kip1 levels and Cdk2 activity. Interestingly, the pattern of TPA-induced changes in HA1 and HD12 cells resembled that in non-endomitotic K562 cells with the exception that the ESG-expressing cells demonstrated a more dramatic down-regulation of retinoblastoma protein. These data suggest that a profound inhibition of the G1/S transition in TPA-treated cells occurs in the presence of Escargot.

To investigate in greater detail whether Escargot was affecting the maintenance of expression of G1/S regulators during TPA-driven differentiation, a time course bivariant flow cytometric analysis was performed. Prior to TPA treatment, cyclins E and A were present in G1/S and S/G2 cells, respectively, as expected for exponentially growing cells (Fig. 4b, t = 0). Subsequent to the addition of TPA, it appeared that the level of both cyclins progressively declined for the first 24 h and that no cells expressed the proteins. In fact, 48 h after differentiation, HA1 and HD12 lacked nuclear cyclins E and A, as was also observed in K562 cells. In contrast, polyploid HELc cells maintained waves of expression of the G1/S cyclins (Fig. 4b). These results suggest that the cell populations that are entering S and M phases at the time of TPA treatment respectively progress through replication and mitosis during the first hours of differentiation, but that no further G1/S transition takes place in escargot-expressing HEL cells.

**Escargot Affects the Transcriptional Regulation of Cyclin A**—We have previously observed that TPA inhibits cyclin A expression in non-endomitotic megakaryoblastic cells (e.g. K562) and that ectopic expression of cyclin E enables these cells to reestablish cyclin A expression and, as a consequence, to undergo endomitotic replication (11). Therefore, a possible target for the action of Escargot could be the transcriptional regulation of cyclin A gene expression. To test this, HELc, HA1, and HD12 cells were transiently transfected with the cycA(--875)luc construct, which contains the luciferase reporter gene under the control of sequences from --875 to +37 base pairs of the human cyclin A promoter. Promoter activity was then assessed in the presence and absence of TPA. TPA. 30 μg of total protein extract from cells exponentially growing (−) or treated with TPA for 48 h (+) was subjected to SDS-polyacrylamide gel electrophoresis and detected by Western blotting with antibodies against the proteins indicated to the right of the upper panel. The lower panel shows the Cdk2-associated kinase activity assayed after immunoprecipitation from TPA-treated (+) and untreated (−) HELc, HA1, and HD12 extracts in the presence of glutathione S-transferase-retinoblastoma protein (Gst-pRb) and γ-[32P]ATP. b, the diagrams show the results from flow cytometric analysis of HA1 cells either untreated (exponentially growing (E)) or treated with TPA for 6, 12, 24, and 48 h (upper panel) and of HD12, HELc, and K562 cells untreated or treated with TPA for 48 h (lower panels). Expression of cyclins A and E was detected by immunofluorescence using a FITC-conjugated anti-cyclin A antibody (Cyc A) or anti-cyclin E/BODIPY-FITC-conjugated goat antimouse IgG (Cyc E) (FL1, vertical axis, linear scale). Total DNA content was monitored by propidium iodide staining (FL2, horizontal axis, linear (in) scale). The positions of cells stained in parallel with the isotype control are indicated by the overlaid polygon.
The data presented here extend this evidence and indicate re-replication control when expressed in adult mammalian establishment of endocycles in early development (19). Escargot and murine Snail factors in the Drosophila evidence had already demonstrated an inhibitory role for cytic polyploidization. Previous genetic and biochemical evidence indicate that human cells contain one or more nuclear endomitotic K562 cells and may involve effects at the level of the regulation of cyclin A gene expression.

**Human Proteins That Bind to the Escargot DNA-binding Site Can Be Detected in Non-endomitotic Cells**—The phenotypic similarities between Escargot-expressing HEL cells and non-endomitotic K562 cells suggested that endogenous Escargot-like proteins could be present in these latter cells. To test this possibility, electrophoretic mobility shift assays were performed on nuclear extracts isolated from untreated or TPA-treated HEL and K562 cells. Under the conditions used to assess ectopic escargot expression in transfected cells, no specific bands could be detected in HELc cells (Fig. 1a) or K562 cells (data not shown). However, when 2–3 times higher amounts of protein extract were used, a major complex could be detected in both HEL and K562 cells (Fig. 6, upper left panel). The specificity of the binding was estimated by competition with an excess of unlabeled EBE or of EBE* (Fig. 6, lower panels). A similar binding pattern was detected in a variety of human cell types, including HeLa (epithelial), and U2OS (osteosarcoma) cells (Fig. 6, upper right panel). Perhaps most significantly, the amount of this specific complex was much higher in electrophoretic mobility shift assays performed with extracts from K562 and the other cells analyzed compared with those using HEL extracts. Interestingly, the amount of protein able to form this specific complex with EBE remained constant, or even increased, in TPA-treated K562 cells, whereas it almost disappeared in differentiated endomitotic HEL cells. These data indicate that human cells contain one or more nuclear EBE-binding proteins. They also suggest that the presence of endogenous EBE-binding proteins, like ectopically expressed escargot in HEL, is not compatible with establishment of megakaryocytic endomitotic cycles.

**DISCUSSION**

In this study, we have shown that constitutive expression of the transcriptional repressor Escargot inhibits megakaryocytic polyploidization. Previous genetic and biochemical evidence had already demonstrated an inhibitory role for Drosophila Escargot and murine Snail factors in the establishment of endocycles in early development (19–21). The data presented here extend this evidence and indicate that the Drosophila protein retains its specific function in re-replication control when expressed in adult mammalian cells. The inhibition of endomitosis is also observed when mouse Snail is ectopically expressed in HEL cells, pointing to a general role of certain members of the Snail family in the control of ploidy maintenance. The cloning of a human homolog, SnaH, has recently been reported (29). It could then be speculated that Escargot is affecting target promoters physiologically controlled by the endogenous protein, given the degree of homology between human and mouse proteins (overall 83%, reaching >95% identity in the DNA-binding region). Although the binding elements that SnaH appears to preferentially recognize are not coincident with the ones shown for its mouse homolog or Escargot (29), it is important to bear in mind that these factors could bind to variants of the sequences tested by in vitro random selections. A putative target of Escargot relevant to megakaryocytic endomitosis could be cyclin A: we have shown here that its expression is down-regulated in escargot-expressing cells and that the transcriptional activity of its proximal promoter region is significantly reduced in these cells. Three E boxes that could account for a repressive effect of Escargot/SnaH on the transcription of the cyclin A gene are present in the proximal promoter region that was present in our cyclin A promoter-reporter construct (CACCCTG, CATATG, and CAACTG at −538, −469, and −387). Although none of these sites corresponds to the consensus Escargot-binding element (19), it could be possible that the overexpressed heterologous protein binds in vivo to one or more of them. Work is in progress to investigate whether cyclin A is one of the putative Escargot target genes that mediates its effect on endoreplication and to determine whether SnaH is physiologically relevant to megakaryocytic polyploidization.

\[ ^2 \text{N. Vilaboa and C. Calés, unpublished observation.} \]
Another interesting point to take into account is that the megakaryocytic endomitotic cycle presents substantial differences compared with embryonic endocycles. Although the latter consist of successions of DNA duplication and short “gap” phases in which G2/M cyclins are absent (1, 22), megakaryocytes complete the G2 phase and enter an abortive mitosis lacking karyo- and cytokinesis (23). However, despite these significant differences, our results suggest that mechanisms similar to those inhibited by Escargot in embryonic cells may account for polyploidy in these highly differentiated adult cells.

In all the systems analyzed to date, polyploidization is accompanied by maintenance of an active G1/S transition and down-regulation of the mitotic machinery (21, 24–27). In megakaryocytic endomitosis, the G1/S transition takes place in the presence of cyclin A and of active, albeit reduced, cyclin B-Cdc2 complexes (3, 5, 10, 11, 28). Here we show that the G1/S transition machinery, previously implicated in the establishment of endomitosis in TPA-treated megakaryoblastic cells, is inhibited in escargot-expressing cells. Thus, Cdk2 activity is not detected in the differentiating escargot-expressing HEL cells, and this could result in the down-regulation of cyclin A. In this respect, our data slightly differ from previous interpretations that Escargot/Snail specifically interfere with G2/M machinery (20, 21). Thus, the inhibitory effect of Escargot and Snail was described to affect the obligate down-regulation of mitotic Cdc2 and cyclins A and B that occurs in embryonic endocycles (20, 21, 26, 27). We have also observed lower levels of cyclin B and also of Cdc2 in escargot-expressing cells, but our interpretation is that the poor expression of these G2/M factors is an indirect consequence of the majority of cells not proceeding beyond G1. We believe that the key point at which Escargot prevents HEL cells from proceeding into endomitotic cycles lies in the regulation of the G1/S machinery, as a profound down-regulation of cyclin E, Cdk2, and also of retinoblastoma protein occurs in the overexpressing cells.

An additional observation is that the expression of escargot in HEL cells results in a phenotype that is strikingly reminiscent of non-endomitotic K562 cells. Hence, escargot-expressing cells respond to TPA by up-regulating megakaryocytic and concomitantly down-regulating erythroidic markers, but do not undergo polyploidization (11). This is also consistent with the idea that Escargot-like proteins could be playing a determinant role in megakaryocytic differentiation. It can then be hypothesized that K562 cells do not enter endomitosis because of the presence of a protein functionally equivalent to Escargot, which would then be down-regulated in differentiating HEL cells. Ectopic expression of escargot in HEL cells might mimic an endogenous factor by interfering with the establishment of endomitotic cycles in these cells. The fact that E2-binding proteins are present in TPA-treated K562 cells but absent in HEL cells suggests that this could be the case. Although further work is needed to determine whether the E2-binding activities that we have detected correspond to Snail factors or to another class of DNA-binding proteins, the evidence presented in this study suggests that megakaryocytic endomitotic differentiation could require the down-regulation or inhibition of such E2 box-binding factors.

Acknowledgment—We thank Dr. Rafael Bornstein for help in morphological characterization of cells.

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