Regulation of Calreticulin Expression during Induction of Differentiation in Human Myeloid Cells

EVIDENCE FOR REMODELING OF THE ENDOPLASMIC RETICULUM*

Received for publication, May 29, 2002
Published, JBC Papers in Press, June 13, 2002,DOI 10.1074/jbc.M205269200

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Induction of differentiation of HL-60 human myeloid cells profoundly affected expression of calreticulin, a Ca\(^{2+}\)-binding endoplasmic reticulum chaperone. Induction with Me\(_2\)SO or retinoic acid reduced levels of calreticulin protein by \(-60\%\) within 4 days. Pulse-chase studies indicated that labeled calreticulin decayed at similar rates in differentiated and undifferentiated cells (t\(_{1/2}\) \(-4.6\) days), but the biosynthetic rate was \(<10\%\) of control after 4 days. Differentiation also induced a rapid decline in calreticulin mRNA levels (90% reduction after 1 day) without a decrease in transcript stability (t\(_{1/2}\) \(-5\) h). Nuclear run-on analysis demonstrated rapid down-regulation of gene transcription (21% of control at 2 h). Differentiation also greatly reduced the Ca\(^{2+}\) content of the cells (25% of control), although residual Ca\(^{2+}\) pools remained able to mediate thapsigargin, ionomycin, and inositol trisphosphate. Progressive decreases were also observed in levels of calnexin and ERp57, whereas BiP/GRP78 and protein disulfide isomerase were only modestly affected. Ultrastructural studies showed a substantial reduction in endoplasmic reticulum content of the cells. Thus, terminal differentiation of myeloid cells was associated with decreased endoplasmic reticulum content, selective reductions in molecular chaperones, and diminished intracellular Ca\(^{2+}\) stores, perhaps reflecting an endoplasmic reticulum remodeling program as a prominent feature of granulocytic differentiation.

Calreticulin is a major Ca\(^{2+}\)-binding protein present in the lumen of the endoplasmic reticulum (ER) of virtually all cell types (1–3). The cDNA sequence predicts an \(-47\)-kDa acidic protein with a zonal structure featuring a globular N-terminal domain, a proline-rich P domain, and a highly acidic C domain terminating in a KDEL motif, the ER retention signal (3, 4).

* This work was supported by a grant from the Medical Research Service of the United States Department of Veterans Affairs. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: ER, endoplasmic reticulum; IP\(_3\), inositol 1,4,5-trisphosphate; IP\(_{3R-1,2,3}\), types 1, 2, and 3 receptors for IP\(_3\); NFDM, non-fat dry milk; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; SERCA, sarco-endoplasmic reticulum calcium ATPase.

Biophysical studies show calreticulin to be a highly asymmetric molecule consistent with this predicted three domain structure. Calreticulin binds Ca\(^{2+}\) with both high and low affinities and with high capacity. A single high affinity site (K\(_d\) \(-1\) \(\mu\)M) is located within the P domain of the molecule and multiple low affinity sites (K\(_d\) \(-2\) \(\mu\)M, 20–25 mol of Ca\(^{2+}\)/mol of protein) are present in the highly acidic C domain (3, 4). The N domain contains at least one site for binding Zn\(^{2+}\), which appears to involve 4 of the histidine residues in this region (5, 6). Binding of these ions affects the conformation and stability of the protein (7).

Numerous and apparently unrelated functions have been ascribed to calreticulin since it was first identified, but its roles in the regulation of Ca\(^{2+}\) homeostasis and as a molecular chaperone of nascent glycoproteins are the two functions that have been most extensively characterized. Calreticulin appears to regulate intracellular Ca\(^{2+}\) homeostasis through more than one mechanism. The high Ca\(^{2+}\)-binding capacity of calreticulin suggests that it acts as a Ca\(^{2+}\) store or buffer within the ER, and there is evidence that in at least some cell types it is a main source of inositol 1,4,5-trisphosphate (IP\(_3\))-releasable Ca\(^{2+}\) (3, 5, 8, 9). In support of these observations, overexpression of calreticulin in some cells is accompanied by a significant increase in the Ca\(^{2+}\) storage pool, as well as an alteration in IP\(_3\)-mediated Ca\(^{2+}\) release and influx (9, 10). Moreover, the thapsigargin- and ionomycin-sensitive Ca\(^{2+}\) pools were markedly reduced in calreticulin-null mouse embryonic stem cells, defects that were corrected by ectopic expression of calreticulin (11). In contrast, calreticulin-deficient murine embryonic stem cells and fibroblasts exhibited normal levels of IP\(_3\)-releasable Ca\(^{2+}\) (12), suggesting that dependence of Ca\(^{2+}\) release on calreticulin may vary among cell types and signaling pathways. Calreticulin may also regulate Ca\(^{2+}\) levels by direct interaction with the ER uptake mechanism. Luminal Ca\(^{2+}\) released into the cytosol through the IP\(_3\) receptor channel is taken back up into the ER by the sarco-endoplasmic reticulum calcium ATPases (SERCA). One isoform of this family of Ca\(^{2+}\) pumps (SERCA2b) has an additional transmembrane segment and a C-terminal domain that extends into the lumen of the ER and contains a putative N-glycosylation site. In elegant studies using the Xenopus oocyte model, Camacho and colleagues (13, 14) showed that calreticulin inhibited the activity of SERCA2b and altered the temporal and spatial patterns of IP\(_3\)-mediated Ca\(^{2+}\) release. This activity was dependent on Ca\(^{2+}\) concentration and was mediated by the lectin-binding P domain of calreticulin, rather than its high capacity Ca\(^{2+}\)-binding C domain.
Thus, calreticulin may modulate agonist-stimulated Ca\(^{2+}\) mobilization through multiple pathways.

In addition to its role in Ca\(^{2+}\) signaling, calreticulin has an important function as a lectin-like chaperone for newly synthesized glycoproteins (3, 15–17). In this respect, it displays many features in common with calnexin, another ER molecular chaperone. Calnexin and calreticulin share regions of structural homology and show lectin-like selectivity for mono-glucosylated N-linked glycoproteins (16, 18–20). The two proteins differ in topography; calreticulin is a soluble luminal ER protein, and calnexin has a transmembrane segment and a cytoplasmic domain. The interactions of calreticulin with unfolded glycoproteins and with other ER chaperones are Ca\(^{2+}\)-dependent, suggesting a potential functional link between the chaperone and Ca\(^{2+}\)-modulating roles of calreticulin (6, 20, 21). Moreover, the 5'-flanking sequence of the calreticulin gene and the genes for the chaperone proteins BiP/GRP78 and GRP94 show regions of sequence homology, suggesting that they are coordinately regulated (22).

In phagocytic leukocytes, calreticulin serves several important functions. It acts as a molecular chaperone for the enzyme myeloperoxidase (15), a catalytic component of oxygen-dependent microbicidal systems. In addition, membranous structures containing calreticulin, as well as the Ca\(^{2+}\)-ATPase SERCA2b, accumulate in the actin-rich filamentous regions surrounding developing phagocytic vacuoles (23), suggesting involvement of these vesicles in the modulation of Ca\(^{2+}\)-dependent phagolysosomal functions. Calreticulin has also been reported to be released from activated neutrophils (24) and may bind and alter the activity of the Clq component of complement (25). A recent report (26) demonstrates that C1q-calreticulin interactions are involved in the uptake of apoptotic cells by phagocytes.

Because of its importance in the biology and function of myeloid cells, we have investigated the biosynthesis and regulation of calreticulin in this cell type. Our previous studies (27) of the biosynthesis and post-translational processing of calreticulin in the HL-60 and PLB-985 myeloid cell lines showed that the primary translation product undergoes co-translational signal peptide cleavage and post-translational N-linked glycosylation to form the mature molecule. In the current study, we have investigated the transcription and translation of calreticulin and characterized the mechanisms of the dramatic down-regulation of its expression during in vitro induction of myeloid cell differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human myeloid cell lines HL-60 (28, 29) and PLB-985 (30) were grown in RPMI supplemented with 2 mm L-glutamine, penicillin-streptomycin, and 10% fetal bovine serum (Invitrogen) at room temperature for 60 min. After washing, the membranes were incubated in ~5 × 10\(^{5}\) cpm/ml \(^{125}\)I-protein A (Amersham Biosciences) in 5% NFDMP/PBS for 4 h at 4 °C, washed in NFDMP/PBS, and autoradiographed. In some experiments parallel immunoblot analyses were carried out with antibodies to a control protein actin (Sigma, A2668) and the calreticulin protein. Proteins calnexin (StressGen Biotechnologies SPA-725, 1:1,000), BiP/GRP78 (Santa Cruz Biotechnology sc-1051, 1:100), and protein disulfide isomerase (Affinity Bioreagents, MA3-018, 1:32,000), and to the cytoplasmic proteins p47\(^{phox}\) and p67\(^{phox}\) (32). These blots were subsequently incubated in the appropriate horseradish peroxidase-labeled immunopurified anti-immunoglobulin antibodies (Pierce ImmunoPure reagent, 1:1,000) and visualized by superchemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce).

**Protein Biosynthesis**—Biosynthetic labeling of cultured cells was carried out as described previously (15, 27) by using a 30-min preincubation in methionine-free medium followed by pulse-labeling with \(^{35}\)Smethionine (~1000 Ci/mmol, Amersham Biosciences). The duration of pulses and subsequent chases with excess unlabeled methionine are indicated in the text and figure legends. Following labeling, the cells were disrupted in an anti-protease buffer and immunoprecipitated with specific anti-human calreticulin (27). In some experiments, immunoprecipitation was also carried out on aliquots of cell culture medium. Immunoprecipitates were analyzed by SDSPAGE and fluorography of the dried gels, and band intensity was quantitated by scanning densitometry (GS-700, Bio-Rad, Hercules, CA). The Northern Blot Analysis—Poly(A\(^{+}\)) RNA was isolated from the cells using SDS lysis and oligo(T) selection (FastTrack, Invitrogen). The RNA was dissolved in nuclease-free water (Invitrogen) and quantitated by absorption at 260 nm. Samples of RNA (3 μg) were denatured in formamide/formaldehyde buffer at 65 °C for 15 min and then separated by electrophoresis (360 V-h) on 1.2% agarose-formaldehyde gels. Separated RNA was transferred overnight to nylon membranes (Nytran Plus, Schleicher & Schuell) by capillary blotting in 20× SSC and then fixed by UV cross-linking. The membranes were pre-hybridized for 2 h at 42 °C in a solution containing 5× SSC, 5× Denhardt's solution, 0.1% SDS, 50% formamide, 50 mm sodium phosphate, pH 6.8, and 100 μg/ml heat-denatured salmon sperm DNA. Probes used in this study (cloned from an HL-60 cell human cDNA library (33)) were the cDNAs for calnexin (1.9 kb), p47\(^{phox}\) (1.36 kb), p67\(^{phox}\) (2.2 kb), and a Patl restriction fragment of type 1 IP\(_{r}\) receptor (1.5 kb; some sequence in common with types II and III IP\(_{r}\) isoforms). Probes were labeled with \(α\)-\(^{32}\)PdCTP (~3000 Ci/mmol, PerkinElmer Life Sciences) using a random-prime labeling kit (Roche Molecular Biochemicals), heat-denatured, and 10000 pmol added to the membrane in fresh pre-hybridization solution. Membranes were then washed in a stepwise manner with a final stringency wash of 0.2× SSC plus 0.1% SDS at 68 °C for 20 min. Following autoradiography the membranes were stripped by soaking in 50% formamide, 2× SSPE at 65 °C for 1 h, and then re-probed as above with DNA for human β-actin (CLONTECH Laboratories, Palo Alto, CA), which served as a sample-loading control. Quantitation was done by scanning densitometry as above. When indicated, transcripts were also examined in a slot-blot format, using the same probes and hybridization conditions as noted above.

**RNA Stability**—Transcript stability was assessed by addition of actinomycin D (50 μg/ml) to cultures of either undifferentiated or induced cells at 0, 1, 2, 3, 4 and 6 h. At the 6-h time point, poly(A\(^{+}\)) RNA was isolated from all sets of cells and analyzed in a slot-blot format, using the same procedures, probes, and hybridization conditions as noted above. Scanning densitometry was used to determine the time required for a 50% decrease from base-line transcript levels, designated the t\(_{1/2}\).

**Nuclear Run-on Analysis**—All solutions were made using diethyl pyrocarbonate-treated sterile distilled and deionized water. Undifferentiated or induced cells (~5 × 10\(^{5}\) in 5 ml of relaxation buffer) were treated with 2 mm diisopropyl fluorophosphate for 20 min and then disrupted by nitrogen cavitation at 350 pounds/square inch for 20 min at 4 °C with evacuation into relaxation buffer containing 1.25 mM EGTA, all as described previously (34). Following centrifugation at 500 × g for 10 min at 4 °C, the nuclear pellet was resuspended on ice in nuclear wash buffer (50 mM Tris-HCl, pH 8.0, 1.25 mM EGTA, 25 mM KCl, 1 mM spermidine), and an aliquot was placed in 1 μg/ml ethidium bromide for determination of concentration and visualization by fluorescence microscopy. After centrifugation at 10000 × g for 10 min at 4 °C, the nuclei were resuspended at 2 × 10\(^{7}\) cells/ml in storage buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl\(_{2}\), 0.1 mM EDTA, 2 mM dithiothreitol, 40% glycerol). Aliquots were then rapidly frozen in a methanol/dry ice bath and stored in liquid N\(_{2}\). For the RNA labeling reaction, 100 μg of freshly thawed nuclei in storage buffer were mixed with an equal volume of...
Calreticulin Regulation in Myeloid Cells

RESULTS

Induction of Differentiation Reduces the Level and Biosynthetic Rate of Calreticulin Protein

Protein Level—MeSO induces granulocytic differentiation of myeloid cell lines (29). To determine the effect of differentiation on calreticulin protein level, HL-60 cells were incubated for 4 days in complete medium supplemented with 1.25% MeSO; aliquots were removed at 0, 2, and 4 days, and cell lysates were analyzed by immunoblotting with monospecific rabbit anti-human calreticulin (27). HL-60 cell calreticulin protein levels decreased over time (Fig. 1A), with quantitative analysis of 5 independent experiments (Fig. 1A, table inset) indicating more than a 50% decline at 4 days. In a separate experiment, 4 different sets of HL-60 cells were analyzed in parallel at 0 and 4 days after MeSO addition (Fig. 1B). There was a consistent decrease in calreticulin levels to a mean of 38.8% (±3.7, p < 0.001) of the zero time controls.

Protein Catabolic Rate—Possible mechanisms that could account for the observed decreases in steady-state calreticulin levels accompanying the induction of differentiation were an increase in the catabolic rate or a decrease in the biosynthetic rate. To determine whether differentiation influenced the rate of disappearance of calreticulin, undifferentiated and 4-day MeSO-induced HL-60 cells were metabolically pulse-labeled with [35S]methionine for 30 min and then chased in fresh complete medium with unlabeled methionine for up to 7 days. For the MeSO-induced cells, the chase medium included Me2SO, although essentially similar results were obtained if without Me2SO, were analyzed in parallel. Quantitation by densitometry indicated that the mean calreticulin content of the 4-day MeSO cells was 38.8% (±3.7; p < 0.001) of control cells cultured in the absence of MeSO. CRT, calreticulin; rhCRT, recombinant human calreticulin marker.

Fig. 1. Immunoblot analysis of calreticulin content of HL-60 cells during MeSO-induced differentiation. A, HL-60 cells were cultured in the presence of 1.25% MeSO for 0, 2, or 4 days as indicated, and lysates of 10^7 cells were separated by SDS-PAGE and blotted to nitrocellulose, and calreticulin was detected with a monospecific polyclonal antibody. The photograph illustrates a representative blot, and the table shows the means (±S.E.) of five independent experiments quantitated by densitometry. B, the experiment was performed as for A, except that five replicate cultures of HL-60 cells, cultured with or without MeSO, were analyzed in parallel. Quantitation by densitometry indicated that the mean calreticulin content of the 4-day MeSO cells was 38.8% (±3.7; p < 0.001) of control cells cultured in the absence of MeSO. CRT, calreticulin; rhCRT, recombinant human calreticulin marker.
Fig. 2. Analysis of the catabolic rate of calreticulin by pulse-chase labeling and immunoprecipitation. HL-60 cells were cultured for 4 days in the presence or absence of Me2SO as in Fig. 1. They were pulse-labeled with [35S]methionine for 30 min and then chased in fresh complete medium with unlabeled methionine for 7 days, and aliquots were removed at intervals, as indicated, for immunoprecipitation, SDS-PAGE, and fluorography. Each lane contained the immunoprecipitate from cells that were pelleted from a fixed volume aliquot of the culture, thereby avoiding the potentially confounding effect of 35S-calreticulin dilution during cell division. The content of calreticulin at each point is indicated as a percent of the zero time value, based on densitometric quantitation. The half-life of calreticulin by this method was 4.5 or 4.6 days for untreated or Me2SO-treated cells, respectively. The study shown is representative of three independent experiments. CRT, calreticulin.

Protein Biosynthetic Rate—In view of the lack of any change in the catabolism of calreticulin to account for its decreased levels in differentiated cells, we next investigated whether the biosynthetic rate was changed in response to Me2SO-induced differentiation. Parallel experiments were also carried out with other differentiating agents. Thus, HL-60 cells were cultured in Me2SO or retinoic acid to induce granulocytic differentiation or in PMA or 1,25-dihydroxyvitamin D3 to induce monocytic differentiation. At intervals, aliquots of cells were taken for biosynthetic pulse labeling with [35S]methionine, immunoprecipitation, and SDS-PAGE analysis (Fig. 3). Induction of differentiation by each agent decreased the amount of nascent calreticulin labeled with [35S]methionine, indicating reductions in the biosynthetic rate. Similar results were observed in studies on PLB-985 cells (data not shown). The effect was progressive over time and showed differences in rate and extent with the various differentiating agents. Initially, the effect was most rapid in the Me2SO-treated cells, which exhibited a reduction of calreticulin synthesis to 36% of zero time levels after only 1 day of treatment. In 4 replicate experiments, the mean normalized values for calreticulin biosynthesis rates (relative to zero time controls) after 1, 2, and 4 days of culture in Me2SO were 44.2 (p < 0.05), 33.8 (p < 0.01), 9.5 (p < 0.001), and 2.0% (p < 0.001), respectively. These experiments clearly indicate that the reduction in calreticulin protein levels observed during differentiation of myeloid cells resulted largely from a major decrease in the rate of calreticulin biosynthesis.

Induction of Differentiation Reduces Calreticulin mRNA Level and Transcriptional Rate

Transcript Level—The studies described above showed that the induction of differentiation of HL-60 cells by a variety of agents resulted in a marked reduction in the biosynthesis of calreticulin. To determine the effect of myeloid cell differentiation on calreticulin mRNA levels, Northern analysis was carried out on the poly(A)+ RNA from cells induced to differentiate (Fig. 4). Calreticulin mRNA was highly expressed in untreated cells but was markedly reduced in both HL-60 and PLB-985 cells within 1 day of Me2SO treatment, and further reduction was seen after 2 days (Fig. 4A). Following normalization to the levels of β-actin mRNA, it was estimated that Me2SO treatment induced an approximate 80% reduction in the level of calreticulin transcripts within 1 day. In 6 replicate experiments, the mean normalized values for calreticulin message levels (relative to zero time controls) after 1, 2, and 4 days of culture in Me2SO were 19.6, 16.2, and 9.1%, respectively (p < 0.001 for each).

Differential expression of calreticulin in myeloid cells was accompanied by the induction of a number of myeloid-specific genes, the products of which carry out essential functions in the terminally differentiated granulocytic cell. Two such genes are p47phox and p67phox, cytosolic components of the phagocyte NADPH oxidase (35). To place the differentiation-induced changes in calreticulin mRNA in context, they were compared with those for p47phox and p67phox in HL-60 cells treated with Me2SO (Fig. 4B). The cellular level of calreticulin mRNA was again rapidly reduced, whereas the p47phox and p67phox transcripts increased from undetectable levels in untreated cells to maximal expression after 2–4 days of Me2SO treatment. Parallel studies were done comparing calreticulin with the type 1 IP3 receptor, a protein that, like calreticulin, is localized to the endoplasmic reticulum and is involved in calcium release (Fig. 4C). We observed that both Me2SO and retinoic acid induced a 3–4-fold increase in IP3R mRNA within 1 day of treatment, whereas calreticulin mRNA was again significantly reduced.

The detailed kinetics of the effects of various inducing agents on calreticulin mRNA expression in HL-60 cells were examined in a slot-blot hybridization assay. RNA was isolated from the cells at 0, 3, 6, 9, 12, 24, and 48 h after initiation of treatment.
and probed for calreticulin and β-actin. After scanning densitometry, calreticulin mRNA was normalized to β-actin levels, and results were expressed as a percentage of the initial value (Fig. 5). Me2SO treatment produced the most rapid reduction (50% in 5 h) of calreticulin mRNA (as was observed for protein synthetic rates, see Fig. 3). Retinoic acid and PMA were also very active, reducing the mRNA levels by 50% in 10 h, whereas 1,25-dihydroxyvitamin D₃ was less active in down-regulation of calreticulin transcripts (50% reduction in 25–30 h).

Transcript Stability—Possible mechanisms that could account for the observed decreases in steady-state levels of calreticulin transcripts accompanying the induction of differentiation were a decrease in transcript stability (i.e., an increase in the catabolic rate) or a decrease in the transcriptional rate. To determine whether calreticulin mRNA stability was affected by Me2SO treatment, studies were carried out using actinomycin D as an inhibitor of transcription. HL-60 cells were cultured in Me2SO for 0, 1, or 2 days and then incubated in fresh medium; actinomycin D was added at various times up to 6 h (see "Experimental Procedures"). At the end of these incubations, poly(A)⁺ RNA was extracted, and slot-blot hybridization analyses were carried out. Levels of calreticulin and β-actin mRNA were expressed as a percentage of the levels at 0 time, and t₁/₂ was calculated from these values. Because the t₁/₂ averaged between 3 and 5 h, analysis focused especially on the 4-h time point. Treatment of the cells with Me2SO resulted in significant and similar increases in the normalized 4-h transcript levels for both calreticulin and actin (Table I), indicating stabilization of these mRNAs in Me2SO-induced HL-60 cells. Thus, the down-regulation of calreticulin mRNA in differentiating HL-60 cells could not be explained by increased transcript catabolism. Indeed, enhanced mRNA stability provided some counter-balance to decreased transcription (see below).

Transcriptional Rate—To determine whether reduction in the rate of gene transcription was responsible for the rapid down-regulation of calreticulin mRNA in differentiating HL-60

Fig. 4. Northern hybridization analysis of calreticulin transcript levels during myeloid cell differentiation. A, HL-60 or PLB-985 cells were cultured in the presence of 1.25% Me₂SO for 0, 1, or 2 days, as indicated. Poly(A)⁺ RNA was isolated, separated (3 μg/lane) on 1.2% agarose-formaldehyde gels, transferred, and probed for calreticulin mRNA, followed by stripping and probing for β-actin, which was used as a loading control. After densitometric quantitation, the content of calreticulin transcripts was corrected for actin and expressed as a percent of the zero time values. The studies shown are representative of five independent experiments for HL-60 cells and two experiments for PLB-985 cells. B, HL-60 cells were cultured and analyzed as in A, except that the blots were also probed for p47phox and p67phox. The data for calreticulin mRNA levels are expressed in the same fashion as above. The study shown is representative of two independent experiments. C, HL-60 cells were cultured and analyzed as in A, except that differentiation was induced with either Me₂SO (Me) or retinoic acid (R) for 1 or 3 days, as indicated, and the blots were also probed for type 1 IP₃ receptor (IP₃R). The data for calreticulin and IP₃R mRNA levels are expressed in the same fashion as above. The study shown is representative of two independent experiments. CRT, calreticulin.
Table I

| Time in Me₂SO (days) | Transcription level (4 h after actinomycin D) | T½ | Actin |
|----------------------|---------------------------------------------|-----|-------|
|                      | Calreticulin | Actin | Calreticulin | Actin |
| 0                    | 27.3 ± 3.9* | 35.4 ± 9.8 | 3.1 ± 0.2* | 3.3 ± 0.4 |
| 1                    | 60.5 ± 5.0 | 38.8 ± 10.3 | 4.7 ± 0.5 | 3.5 ± 0.2 |
| 2                    | 62.9 ± 6.6 | 57.4 ± 21.5 | 5.0 ± 0.6 | 4.8 ± 0.7 |

* Percent of control; mean ± S.E. for a total of 4–6 independent experiments.

**Induction of Differentiation Reduces Ca²⁺ Storage Pools**

Current evidence indicates that calreticulin functions as a principal ER store or buffer for intracellular Ca²⁺. To determine whether the decrease in calreticulin content observed during differentiation of HL-60 cells was accompanied by a change in Ca²⁺ storage capacity, cells were labeled to isotopic equilibrium with ⁴⁵Ca²⁺ for 54 h in the presence or absence of Me₂SO. Differentiation had a profound effect on Ca²⁺ content, reducing total cell-associated ⁴⁵Ca²⁺ by about 75% (Fig. 7). Nonetheless, the Ca²⁺ pool in the differentiated cells, although greatly reduced, was sensitive to both thapsigargin and ionomycin treatment, suggesting that differentiation resulted in a quantitative effect on intracellular Ca²⁺ stores, rather than an alteration in the nature of the stores themselves. This was supported in further studies, where it was observed that the fraction of total cellular Ca²⁺ released after stimulation with the physiologic agonist IP₃ was similar in undifferentiated and differentiated cells (data not shown).

**Induction of Differentiation Causes Selective Reductions in Levels of ER Proteins**

To investigate the effect of differentiation on other ER resident proteins besides calreticulin, and to control for the possibility of a generalized Me₂SO-induced impairment of protein homeostasis, the effect of Me₂SO treatment on a number of other proteins was investigated (Fig. 8). Among the ER proteins examined there was a hierarchy of responses. Calreticulin was consistently and strongly down-regulated as shown above, and calnexin levels were very similarly reduced over the course of 6 days of differentiation. ERp57 exhibited substantial down-regulation, although more slowly and to a lesser extent than for calreticulin and calnexin (Fig. 8). In contrast, protein-disulfide isomerase and Bip/GRP78 remained unchanged or decreased only modestly. In the same experiments, the levels of the NADPH oxidase cytosolic components p67phox and p47phox increased markedly during differentiation, and the levels of actin were essentially unchanged. Thus, the induction of granulocyte differentiation in HL-60 cells was associated with selective reduction of a subset of ER chaperone proteins.

**Induction of Differentiation Reduces ER Content**

The decrease in expression of the ER resident proteins calreticulin, calnexin, and ERp57 suggests that the induction of differentiation in HL-60 cells is associated with a change in the subcellular ER compartment itself. To investigate morphological changes in cellular ultrastructure, HL-60 cells were treated with Me₂SO for up to 6 days and then fixed and processed for transmission electron microscopy. Marked changes in morphology consistent with the granulocytic maturation/differentiation of the cells were observed in both the nuclear and extranuclear compartments. The control untreated cells displayed a rounded morphology, with large non-lobulated nuclei containing prominent nucleoli and dispersed nuclear chromatin (Fig. 9, A–C). The extranuclear compartment of the untreated cells contained an abundant network of rough ER.
contrast, the nuclei of cells treated with Me₂SO for 6 days demonstrated a pyknotic accumulation of nuclear chromatin and marked lobulation and ER profiles were much less prevalent than in undifferentiated cells (Fig. 9, D–E).

**DISCUSSION**

Calreticulin is a widely expressed and highly conserved Ca²⁺-binding ER protein. It is essential for normal embryonic development (36) and participates in a wide range of cellular functions, including Ca²⁺ homeostasis and signaling, nascent protein folding, regulation of steroid-sensitive genes, and modulation of cell adhesion (1, 3, 4). Despite these important roles, relatively little is known about the regulation of expression of the calreticulin gene and how such regulation may affect cell function.

Pharmacologic depletion of ER Ca²⁺-stores has been shown to up-regulate expression of calreticulin in HeLa and NIH 3T3 cells through a mechanism involving transcriptional activation (37–39). Transcription of the calreticulin gene is also activated by heat shock in several cell types (37, 40, 41). Redox mechanisms may be involved in calreticulin regulation, although whether oxidant stress results in decreased (42) or increased (43) expression appears to depend on the experimental system employed. Several examples of tissue-specific regulation of calreticulin expression have been reported. Activation of T lymphocytes by concanavalin A was associated with increased calreticulin content, primarily due to increased protein stability (52).

However, at the cellular level, the two model systems reported to date exhibited divergent patterns. In the myogenic cell line L6, it was determined that calreticulin was not regulated during differentiation to mature myotubes (51). In contrast, differentiation of NG-108-15 neuroblastoma-glioma cells was associated with increased calreticulin content, primarily due to enhanced protein stability (52).

The current report represents the most complete and systematic analysis to date of the effects of cell differentiation on rates of calreticulin transcription and translation, as well as the catabolism of calreticulin mRNA and protein. We made use of two well characterized human myeloid cell lines, HL-60 and PLB-985, and four differentiation-inducing agents, two of which lead to the granulocytic pathway and two of which lead to the monocyctic pathway. Given the substantial evidence for important functional roles of calreticulin in myeloid cells (15, 23–25), these models are expected to be biologically relevant. Initially, we observed that myeloid differentiation led to consistent and major decreases in the cellular content of calreticulin protein. Pulse-labeling experiments then demonstrated that the decreased calreticulin levels could not be ascribed to an increased catabolic rate of the protein in differentiated cells. Interestingly, calreticulin proved to be an exceedingly stable protein, at least in the myeloid cell lines tested, a finding that is in keeping with earlier qualitative observations in a muscle cell line (53). In our quantitative studies the calculated fractional catabolic rate was only 13% per day. Completing our
analysis at the protein level were pulse-labeling studies that demonstrated dramatic decreases in the rate of calreticulin biosynthesis during myeloid cell differentiation. Comparison of the synthetic and catabolic rates indicated that the decline in steady-state calreticulin levels due to the rapid decrease in synthesis was partially buffered by slow catabolism. For example, at a time during differentiation when the biosynthetic rate was only about 10% of the base-line rate, the protein content was still maintained at about 40% of base-line levels.

Turning to analysis of calreticulin mRNA, we found that myeloid differentiation resulted in very rapid decreases in transcript levels, approximately in parallel to the observed decreases in synthesis of the protein. This effect did not represent a generalized decrease in mRNA levels, because we found concomitant increases in transcripts for the NADPH oxidase components p47^phox and p67^phox. Interestingly, as calreticulin transcripts decreased, those for the type I IP3 receptor actually increased. Up-regulation of the IP3 receptor under these circumstances, although well described previously (54, 55), is curious, given the co-localization of the receptor with calreticulin in the ER Ca2+ storage organelles and the shared functional participation of the two proteins in stimulus-mediated Ca2+ release. The decrease in calreticulin mRNA during differentiation of myeloid cells could not be ascribed to increased catabolism of the transcripts. Indeed, calreticulin mRNA, as well as actin mRNA, exhibited an increased half-life in differentiated cells. Finally, we assessed transcriptional rates by nuclear run-on analysis. These experiments demonstrated that transcription of the calreticulin gene decreased by about 80% within 2 h of HL-60 cell exposure to the differentiation-inducing agent, Me3SO. Comparisons with other genes showed similar transcriptional down-regulation of myeloperoxidase versus up-regulation of p47^phox and little or no change in actin. It appears that the extremely rapid decrease in calreticulin transcriptional rate is partly offset by an increase in mRNA stability but that steady-state transcript levels decrease rather quickly once transcription is essentially shut down. Putting together the entire sequence of events for calreticulin from gene to protein, myeloid cell differentiation is associated with very rapid down-regulation of transcription, whereas the impacts on steady-state transcript and protein levels are modulated by increased transcript stability and constitutively high protein stability.

What are the functional consequences of altered levels of calreticulin expression? There are several reports (9, 10, 56, 57) documenting significant effects of calreticulin overexpression on Ca2+ signaling and homeostasis. In general, these studies demonstrated increases in IP3- or thapsigargin-sensitive Ca2+ stores, as well as decreased store-operated (capacitative) Ca2+ entry via plasma membrane channels. Conversely, antisense oligonucleotide-mediated suppression of calreticulin resulted in a decrease in IP3-dependent release of Ca2+ stores (58). Calreticulin-deficient mouse embryonic fibroblasts exhibited decreased total Ca2+ stores and proportionately decreased release by thapsigargin, ionomycin, or IP3 (11). Consistent with these studies, the decreased cellular content of calreticulin that we observed during the physiologic process of myeloid cell differentiation correlated with a commensurate decrease in the Ca2+ stores. Yet despite this reduction, the residual Ca2+ stores remained sensitive to thapsigargin, ionomycin, and IP3.

In addition to the differentiation-induced change in the size of the intracellular Ca2+ pools that we have described here, there is evidence that myeloid cell differentiation is also accompanied by a qualitative change in the mechanisms that regulate agonist-mediated Ca2+ signaling and homeostasis. Induction of differentiation in HL-60 cells was reported to result in a redistribution of the subtypes of both the IP3 receptors that regulate the release of Ca2+ from the internal stores and the SERCA proteins that are responsible for refilling the depleted stores (59). A steady decline in the expression of the dominant SERCA2b isoform was observed, together with a rapid increase of the SERCA2aLIM (SERCA3b) isoform. SERCA3b has a lower affinity for Ca2+ than SERCA2b and presumably displays slower kinetics in refilling Ca2+ stores, resulting in a more sustained elevation of cytosolic Ca2+ following stimulation. With respect to IP3 receptors, differentiation of HL-60 cells resulted in increased expression of the type 2 receptor (IP3-R2) compared with either the type 1 or type 3 isoforms. IP3-R-2 has a higher binding affinity for IP3 than either IP3-R-1 or IP3-R-3 (60), and recent functional studies indicated that, unlike IP3-R-1, IP3-R-2 is not inhibited by high cytosolic concentrations of Ca2+ (61). Thus, the increased expression of SERCA3b and IP3-R-2 during myeloid differentiation would be expected to result in increased sensitivity to Ca2+ -mobilizing agonists, together with a more sustained response. This is consistent with the well known properties of terminal differentiated mature granulocytes as highly reactive and motile cells that respond rapidly to external stimuli by chemotaxis, phagocytosis, and the release of enzymes and inflammatory mediators.
The rate of reduction of SERCA2b in differentiating HL-60 cells reported earlier (59) is similar to the rate of reduction in the levels of calreticulin that we have described here. Because it has been shown that both calreticulin and calnexin influence SERCA2b activity (13, 14), it may well be that these proteins form a functional unit and occupy the same ER sub-compartment. It is tempting to speculate that their reduced expression during differentiation reflects selective attrition of this sub-compartment that may account for at least part of the reduced total cell Ca\(^{2+}\) content that we observed. In support of this possibility is evidence that the SERCA2b and SERCA3 Ca\(^{2+}\) transport proteins are associated with different functional pools of intracellular Ca\(^{2+}\) stores, the SERCA3b pool being more sensitive to release by IP\(_3\) (62, 63). Therefore, it is possible the differentiation-induced reduction of SERCA2b may be accompanied by a reduction in the size of its associated Ca\(^{2+}\) pool.

In addition to its Ca\(^{2+}\) storage role, calreticulin functions as an ER molecular chaperone for nascent glycoproteins (16–19). In myeloid cells, for example, the microbical protein myeloperoxidase interacts with calreticulin during its biosynthesis (15). The implications for protein synthesis and processing of the differentiation-induced decrease in calreticulin levels in myeloid cells are uncertain. Of note, a recent report by Nakamura and colleagues (11) suggests malfolding of the bradykinin component of the differentiation-induced decrease in calreticulin levels in myeloid cells. Clearly, protein synthesis continues during myeloid cell differentiation, albeit with an altered program of gene expression. The gp91 \(^\text{phox}\) component of the phagocyte NADPH oxidase is a good example of a glycoprotein that is strongly up-regulated during differentiation, although whether it interacts with calreticulin has not been reported. Interestingly, it was found recently that the selection of a particular chaperone pathway by a nascent glycoprotein was partly dependent on the position of the N-linked glycan. If the glycosylation site was within –50 residues of the N terminus, the glycoprotein interacted preferentially with calreticulin and calnexin. In contrast, nascent glycoproteins with N-linked glycans more distant from the N terminus interacted initially with BiP/GRP78, a member of the Hsp70 family, and only post-translationally with calreticulin and calnexin (64). In the case of gp91 \(^\text{phox}\), the N-linked glycosylation sites are all >90 residues from the N terminus, suggesting that it might interact preferentially with BiP/GRP78.

The selective down-regulation that we have demonstrated for calreticulin, calnexin, and ERP57 during cell differentiation may influence the pathways and efficiency of glycoprotein folding in the ER. Our ultrastructural analysis of differentiating HL-60 cells, as well as earlier studies of mature neutrophils (65), support the concept that major remodeling of the ER is an inherent feature of granulocytic differentiation. Whether this ER plasticity is accompanied by specific reprogramming of glycoprotein processing and how alternative chaperones such as BiP/GRP78 and protein-disulfide isomerase, both of which we found to be relatively preserved, may compensate for the losses of calreticulin, calnexin, and ERP57 require further study.

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