Induction of ubiquitin-conjugating enzymes during terminal erythroid differentiation

(erythropoiesis)

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ABSTRACT   A global cellular reorganization occurs during the reticulocyte stage of erythroid differentiation. This reorganization is accomplished partly through programmed protein degradation. The selection of proteins for degradation can be mediated by covalent attachment of ubiquitin. We have cloned cDNAs encoding two ubiquitin-conjugating (E2) enzymes, E2-20K and E2-230K, and found their genes to be strongly induced during the differentiation of erythroblasts into reticulocytes. Induction of the E2-20K and E2-230K genes is specific, as transcript levels for at least two other ubiquitinating enzymes fall during erythroblast differentiation. In contrast to most proteins induced in reticulocytes, E2-20K and E2-230K enzymes are present at strongly reduced levels in erythrocytes and thus decline in abundance as reticulocyte maturation is completed. This result suggests that both enzymes function during the reticulocyte stage, when enhanced protein degradation has been observed. These data implicate regulated components of the ubiquitin conjugation machinery in erythroid differentiation.

In erythroid differentiation, a combination of selective protein synthesis, protein degradation, and organelle extrusion brings about extensive cellular reorganization and dramatic changes in the cellular protein composition (1–3). Similar events characterize terminal differentiation of many other tissues, but the erythroid lineage is distinct in being unusually amenable to biochemical characterization. Strikingly, the end products of erythroid differentiation lack nuclei, mitochondria, ribosomes, lysosomes, endoplasmic reticulum, many cytoplasmic and cytoskeletal proteins, as well as other components present in their progenitors. The elimination of these components is an active process occurring primarily, but not exclusively, in the reticulocyte, the direct precursor to the erythrocyte. Cell nuclei are lost through extrusion, but most cellular proteins appear to be eliminated through intracellular degradation pathways (1).

A major pathway of protein breakdown involves covalent conjugation of proteolytic substrates to a 76-residue protein, ubiquitin (4). Proteins ligated to multiple ubiquitin groups are specifically degraded by the 26S protease (5, 6). Substrates of this pathway include M-, S-, and G_1-phase-specific cyclins (7–10), cyclin-dependent kinase inhibitors (11), transcription factors (12, 13), p53 (14), and a variety of oncoproteins (4, 15, 16). Attachment of ubiquitin to proteins is carried out by the sequential action of three classes of enzymes, ubiquitin-activating enzymes (referred to as E1 or Uba), ubiquitin-conjugating enzymes (E2 or Ubc), and ubiquitin-protein ligases (E3 or Ubr) (4). E1 enzyme forms a thiolester bond with the C terminus of ubiquitin, which is then transferred to a thiol of an E2 enzyme. Although E2 enzymes can transfer ubiquitin

directly to protein lysine residues, E3 enzymes are typically required for recognition of specific proteolytic substrates (4, 14, 17, 18). E2 enzymes are encoded by a multigene family (19). The functions of E2 enzymes are known mainly from studies in yeast; phenotypes of E2-deficient mutants include defects in DNA repair, organelle biogenesis, and the stress response (19). Individual E2 enzymes function in the turnover of distinct sets of protein substrates (8–13, 17, 19), possibly due to specific interactions with E3 enzymes.

Here we describe evidence suggesting a role of ubiquitination in cell differentiation. Genes encoding the ubiquitin-conjugating enzymes E2-20K (20) and E2-230K (21) are shown to be induced specifically in differentiating erythroid cells. However, these enzymes are not major components of the differentiated cell but are present transiently during the course of differentiation. Genes encoding a variety of other E2 enzymes are repressed as the E2-20K and E2-230K genes are induced. Therefore, enhanced protein turnover in reticulocytes does not appear to involve generalized activation of the ubiquitin pathway but rather specific induction of particular conjugating enzymes. The selectivity of ubiquitin-dependent protein turnover is determined primarily by the conjugation step of the pathway (4, 17). The regulation of specific ubiquitin-conjugating enzymes during cell differentiation may thus be expected to change the nature of the preferred targets for proteolysis and may allow differentiating cells to degrade proteins inherited from progenitor cells.

MATERIALS AND METHODS

Reticulocyte Induction and Lysis. Both male and female mice were used, weighing 28–35 g. Reticulocytes were induced by five daily i.p. injections of phenylhydrazine (40 μg/g of body weight), followed by 2 days without injection. Reticulocyte counts typically ranged from 55% to 75%, as estimated from staining with cresyl blue. Cells were pelleted by centrifugation at $\approx$800 $\times$ g and then washed three times with 10–15 vol of ice-cold phosphate-buffered saline (150 mM NaCl/10 mM sodium phosphate, pH 7.4).

Cell Culture. Mouse erythroblasts infected with the anemia-inducing strain of Friend virus cells (FVA) were prepared as described (22). Briefly, female CD_2 F_1 mice were injected with 10^4 spleen focus-forming units of the anemia-inducing strain of Friend virus. After 2 weeks, spleens were harvested from the mice, and immature erythroblasts were isolated by unit gravity sedimentation over a 1–2% gradient of bovine serum albumin (Intergen, Purchase, NY). Cells were cultured in 5% CO_2/air at a density of 1 $\times$ 10^6 cells per ml. The cell culture medium was Iscove's modified Dulbecco's medium (IMDM) (GIBCO/

Abbreviations: Epo, erythropoietin; FVA cells, mouse erythroblasts infected with the anemia-inducing strain of Friend virus; MEL, murine erythroleukemia.
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BRL) containing 30% fetal calf serum (GIBCO/BRL), 0.1% deionized bovine serum albumin, 0.1 mM monothioglycerol, penicillin at 100 units/ml, and streptomycin at 1 mg/ml. A concentrated stock of erythropoietin (Epo) in IMDM was diluted to a final concentration of 1.0 unit/ml in FVA cultures grown in the presence of Epo. Cellular morphology was monitored by staining cells with 3,3-dimethoxybenzidine (23) after they had been cytocentrifuged onto glass slides.

Extraction of Proteins from Cells and Tissues. Washed reticulocytes and erythrocytes were lysed by mixing with 3 vol of ice-cold 1 mM dithiothreitol. The lysate was centrifuged at 100,000 × g for 60 min (5°C), and the supernatant (extract) was stored at −60°C until use. Murine erythroleukemia (MEL) cells were cultured in standard medium, as described (24). Cells were counted, washed several times in ice-cold Hanks' salts buffered with Hepes (pH 7.4), and lysed by mixing in buffer containing 5 mM Tris·HCl (pH 7.6), 6 M urea, 5 mM EDTA, 5 mM N-ethylmaleimide, 1% (vol/vol) Nonidet P-40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and leupeptin and soybean trypsin inhibitor each at 10 μg/ml. After 10 min on ice, the lysate was centrifuged (15,000 × g, 15 min), and the supernatant was stored at −60°C until use. Extracts of FVA cells were prepared similarly to MEL cell extracts, except that urea was omitted from the lysis buffer, and Nonidet P-40 was present at 0.1%.

Individual organs were minced and homogenized in a buffer containing 5 mM Tris·HCl (pH 7.6), 5 mM EDTA, 2 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, leupeptin at 5 μg/ml, and soybean trypsin inhibitor at 10 μg/ml. To minimize erythroid contamination of organs, mice (previously euthanized with CO₂) were subjected to cardiac perfusion with phosphate-buffered saline before excision of organs. Organ homogenates were centrifuged at 800 × g; the supernatant was centrifuged at 100,000 × g for 60 min. The final supernatant was stored at −60°C until use.

Anion-Exchange Chromatography of Extracts. Total protein concentrations in various extracts were determined by using Bio-Rad dye reagent, with bovine serum albumin as standard. Extracts were analyzed directly for E2-25K (see below). Stepwise anion-exchange fractionation was carried out to enrich for E2-20K and E2-230K before analysis, by a modification of a published procedure (24). Soluble extracts were applied to Q-Sepharose, and the loaded columns were washed with 4 vol of buffer containing 20 mM Tris·HCl (pH 7.5), 0.2 mM EDTA, and protease inhibitors as above, followed by 3 vol of the same buffer containing 0.2 M NaCl. Columns were then eluted with 3 vol of the same buffer containing 0.6 M NaCl. After concentration by ultrafiltration, this latter eluate was analyzed for E2-20K and E2-230K (see legend to Fig. 1).

Protein Purification. Recombinant bovine E2-25K (25) and rabbit reticulocyte E2-230K (21) were purified as described. Enzyme concentrations were determined as described above. The murine E2-20K cDNA was cloned into pET-19b and expressed in *Escherichia coli* strain BL21(DE3)/pLysS (26); then E2-20K was purified to electrophoretic homogeneity. Cells were lysed (26), and insoluble material was pelleted and discarded (26). Proteins precipitating between 30% and 50% saturation with ammonium sulfate were collected, dialyzed, and applied to Q-Sepharose, followed by gradient elution as described (20). Pooled E2-20K-containing fractions were chromatographed on Sephacryl-200 in 50 mM MES, pH 6.5. The pooled peak fractions were applied to a Mono fast protein liquid chromatography Q column equilibrated with the same buffer, and eluted with a 0–0.6 M NaCl gradient. E2-20K eluted at 0.3 M NaCl. Fractions were routinely monitored by SDS/PAGE and Coomassie blue staining.

Antibodies. Preparation and affinity purification of antibodies against recombinant E2-25K and a synthetic peptide (CVSDSLFFDDSY) from E2-230K have been described (27).

Antibodies against E2-20K were induced by immunization of female New Zealand White rabbits with the purified SDS-denatured protein as described (28). Antibodies were purified (28) from crude immune serum by affinity chromatography on E2-20K-Sepharose prepared from activated CH-Sepharose (Pharmacia LKB).

Immunoblot Analysis. Protein samples were analyzed by SDS/PAGE (29), using acrylamide concentrations of 8% for E2-230K, 13.5% for E2-20K, and 12.5% for E2-25K. Electrophoretic transfer was done for 3 hr at 5°C as described (28), except that Immobilon-P (Millipore) was used in place of nitrocellulose. All subsequent procedures were done at room temperature. Blots were blocked for 30 min in buffer A (28) and then incubated for 1 hr with specific antibody diluted appropriately in the same buffer. After three short washes in TST buffer (50 mM Tris·HCl, pH 7.6/0.15 M NaCl/0.5% Tween 20), blots were incubated for 1 hr in buffer A containing either secondary antibody [alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad)] or ¹²⁵I-labeled protein A. Blots developed with protein A were washed with TST buffer and autoradiographed. Blots developed with secondary antibody were washed and stained colorimetrically.

RNA Preparation and Analysis. Procedures for RNA extraction were as described (30). Ten micrograms of total RNA was separated on formaldehyde-containing 1% agarose gels (31) and transferred to Nytran filters (Schleicher & Schuell) according to the supplier's recommendations. Filters were prehybridized in 5× standard saline phosphate/EDTA (SSPE) [1× SSPE is 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA]/10× Denhardt's solution/50% (vol/vol) formamide/0.5% SDS/heat-denatured salmon sperm DNA at 100 μg/ml and 42°C (32). The same conditions were used for hybridizations with RNA probes. For oligo-labeled (33) DNA fragments (E2-20K, E2-230K, and 18S rRNA), 2% SDS was used in the hybridization solution. Radiolabeled probe was added to the prehybridization solution at 2 × 10⁶ cpm/ml, and hybridization was done for at least 16 hr. Filters were then washed twice for a total of 30 min in 1× standard saline citrate (SSC)/0.5% SDS at 65°C and 30 min in 55% formamide (vol/vol)/2× SSC/1% SDS at 65°C (32). For DNA probes, the second wash solution was 1% SDS/0.2× SSC. Filters were exposed to Kodak x-ray films or PhosphorImager screens. The E2-20K probe contained sequences from −22 to +381 with respect to the initiator ATG codon. The E2-230K probe was a 1572-bp *Sac* I fragment from the coding region. GenBank accession numbers are U19854 for the E2-20K sequence and U20780 for E2-230K. RNA probes (32) were derived from a synthetic ubiquitin gene (34), a human β-globin cDNA, a human UbcH5 cDNA (35), and a bovine E2-25K cDNA (36). Transcripts were quantitated by using a PhosphorImager.

RESULTS

Tissue-Specificity of E2-20K and E2-230K Expression. Systematic characterization of mammalian ubiquitin-conjugating enzymes has been done primarily in reticulocyte extracts. None of the E2 enzymes of the reticulocyte are known to be specific to these cells or under major developmental control. Here we characterize a pair of E2 enzymes, E2-20K and E2-230K, that show such regulation. To search for tissue-specific E2 enzymes, we raised antibodies to a variety of E2 enzymes purified from reticulocytes and used these antibodies to probe immunoblots loaded with extracts from various murine tissues. E2-20K (20) and E2-230K (21) were found at high levels in reticulocytes and either were undetectable or present at lower levels in other tissues (Fig. 1). In contrast, E2-25K (25, 36, 37) was present at high levels in most tissues (Fig. 1), as observed for several other mammalian E2 enzymes (C.M.P., unpublished data; see also below), but at low levels in reticulocytes (see below).
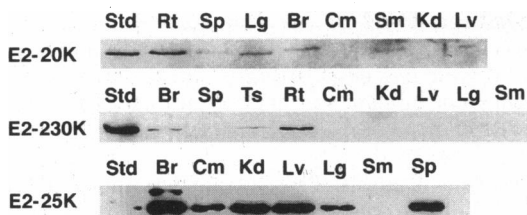
FIG. 1. Tissue specificity of E2 protein expression. Extracts from murine cells or tissues were electrophoresed, transferred to poly(vinylidene difluoride) membranes, and probed with affinity-purified antibodies to E2-20K, E2-230K, or E2-25K, as indicated. The first lane of each panel is a purified quantitative standard (Std). Rt, reticulocyte; Sp, spleen; Lg, lung; Br, brain; Cm, cardiac muscle; Kd, kidney; Lv, liver; Ts, testis; Sm, skeletal muscle. Q-Sepharose fractions derived from 50 μg (E2-20K, E2-230K) or 20 μg (E2-25K) of soluble protein were analyzed from each tissue. Standards: 100 fmol of E2-20K, 100 fmol of E2-230K, and 50 fmol of E2-25K. Lanes in each row are derived from a single blot.

E2-20K and E2-230K proteins were purified from rabbit reticulocytes, and several tryptic peptides from each protein were isolated and sequenced. Peptides from E2-20K displayed sequence similarities to a human E2, UbcH2, for which the cDNA has been previously cloned (38). The tissue specificity of UbcH2 has not been characterized. By screening a murine reticulocyte cDNA library (39) with a UbcH2-derived probe, we cloned the E2-20K cDNA, encoding a protein for which the deduced amino acid sequence was identical to that of UbcH2. E2-230K cDNA was isolated from the same library by immunoscreening. The predicted sequence of E2-230K was not significantly similar to that of any known protein, except in the conserved region surrounding the active-site cysteine of E2 enzymes (19). E2-230K is an unusual member of the E2 protein family because it has abundant conjugative targets in reticulocytes (C.M.P., unpublished data) and is exceptionally large in comparison with other E2 enzymes, most of which are 15- to 30-kDa proteins.

E2-20K and E2-230K probes were used to measure mRNA levels from a variety of murine tissues. In reticulocytes, the major transcripts encoding E2-20K and E2-230K are ≈3.5 and 7 kb, respectively (Fig. 2). In addition, the E2-20K probe detected a less abundant transcript of ≈6.5 kb (Fig. 3). All three transcripts appear to be polyadenylylated (data not shown). The two transcripts detected by the E2-20K probe are presumably derived from the same gene because they are detected (with equivalent relative intensities) by nonoverlapping probes from coding and untranslated portions of the E2-20K cDNA (data not shown). Transcripts encoding E2-20K
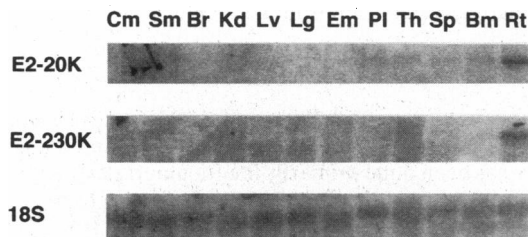

FIG. 2. E2-20K and E2-230K transcripts are expressed preferentially in reticulocytes. Radiolabeled probes derived from E2-20K and E2-230K cDNAs were hybridized to electrophoretically fractionated RNA from various murine tissues. Tissue designations are as in Fig. 1, except for the following: Em, total embryo (day 12); Pl, placenta; Th, thymus; Bm, bone marrow. Shown are the 3.5-kb transcript for E2-20K and the 7-kb transcript for E2-230K; the minor 6.5-kb transcript for E2-20K (see Fig. 3) was not sufficiently abundant to be detected in this experiment. The 18S rRNA bands show that each lane contains approximately equal amounts of RNA. The E2-20K and E2-230K clones will be described in detail elsewhere.

and E2-230K were preferentially expressed in reticulocytes as compared with 11 other tissues (Fig. 2), including spleen and bone marrow, which contain cells in early stages of erythroid differentiation. When more sensitive detection methods were used, we detected transcripts for E2-20K and E2-230K in samples from a variety of nonerythroid cells, consistent with the immunoblotting data of Fig. 1 (unpublished data).

Induction of E2-20K and E2-230K in FVA Cells. The data above suggested that the E2-20K and E2-230K genes might be induced during erythroid differentiation. To test this possibility, we examined the expression of these genes during terminal differentiation of FVA cells (22, 23, 40–42). FVA cells provide a homogeneous population of erythroid progenitors in sufficient numbers that biochemical studies can be done. Unlike MEL cells, which are frequently used to study erythroid differentiation, FVA cells differentiate in the presence of the physiological inducer of erythroid differentiation, Epo. Moreover, the time course of FVA cell differentiation resembles that of normal erythroblasts *in vivo*; most FVA cells differentiate to the reticulocyte stage of development after 48 hr of culture in the presence of Epo. Events that accompany Epo-mediated differentiation of FVA cells include the accumulation of hemoglobin, condensation of nuclear chromatin, a decrease in nuclear size, and the eventual extrusion of nuclei from FVA cells to produce reticulocytes (42). When deprived of Epo in culture, FVA cells eventually undergo apoptotic cell death (43–45).

Both E2-20K and E2-230K transcripts were strongly induced in Epo-treated FVA cells (Fig. 3A). Surprisingly, for E2-230K, the relative rate of mRNA induction in FVA cells was almost indistinguishable from that of globin (Fig. 3B). Both genes were induced ≈16- to 17-fold after 2 days of culture in the presence of Epo. The kinetics of globin induction in these experiments was similar to that reported for FVA cells (40). A pronounced induction of E2-20K mRNA was also seen, with a 10-fold rise in level by day 2 (Fig. 3B). Neither E2-20K nor E2-230K was induced in the absence of Epo (data not shown). The Epo-dependent induction of E2-20K and E2-230K was also apparent at the protein level (Fig. 4A). After 48 hr of culture in the presence of Epo, the level of E2-230K in FVA cells was equivalent to that in reticulocytes, whereas E2-20K was found at 20% of the level in reticulocytes.

Regulation of Other E2 Genes in FVA Cells. As the E2-230K and E2-20K genes were induced, the expression of murine homologs of E2-25K and UbcH5 (Fig. 3), as well as other E2 enzymes (data not shown), was down-regulated. Accordingly, E2-25K protein declined in abundance during differentiation, in contrast to E2-20K and E2-230K (Fig. 4A). Thus, different members of the E2 enzyme family show opposing patterns of regulation. However, E2-25K, and perhaps other E2 enzymes that do not appear to be induced during erythroid differentiation, are nonetheless present in reticulocytes (20), although at a reduced level, and presumably contribute to protein turnover in these cells.

Transient Nature of E2-20K and E2-230K Induction. The induction of E2-20K and E2-230K in reticulocytes suggests that they function during either the reticulocyte or the subsequent erythrocyte phase of differentiation. However, the high levels of E2-20K and E2-230K proteins seen in reticulocytes were not seen in erythrocytes (Fig. 4B), indicating that these proteins are eliminated during a late phase of erythroid maturation, consistent with activity assays (20). The regulation of these E2 enzymes is therefore distinct from that of the many enzymes, transporters, and structural proteins that are synthesized in the reticulocyte and become major constituents of the erythrocyte (1, 46). Thus, E2-20K and E2-230K may represent a separate class of erythroid proteins that do not function in the mature erythrocyte but rather function in the differentiation process that gives rise to it (47).
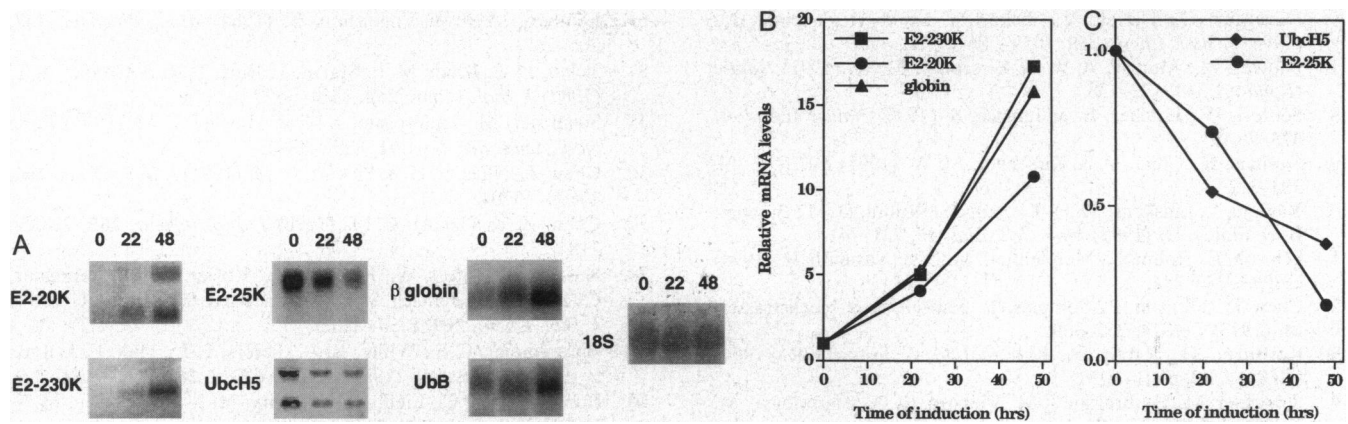
FIG. 3. Induction of E2-20K and E2-230K transcripts during erythroid differentiation. (*A*) RNA was prepared from FVA cells 0, 22, and 48 hr after Epo addition and hybridized with radiolabeled probes as indicated. The polyubiquitin (UbB) gene was induced 4-fold. (*B* and *C*) Hybridization signals from *A* were quantitated and normalized to 18S rRNA. In *B* induction of the 3.5-kb transcript is plotted. This transcript could be quantitated more precisely than the 6.5-kb transcript because of its higher abundance. The 6.5-kb band is a minor band in reticulocytes as well (data not shown).

DISCUSSION

We have found that erythroid differentiation involves major alterations of the ubiquitin-conjugation machinery. The data suggest a specific mechanistic basis for the enhanced protein turnover observed in reticulocytes, which underlies the dramatic changes in cellular protein composition that mark terminal differentiation in the erythroid lineage (1–3). Although E2-20K and E2-230K are thus far the only E2 enzymes found to be induced in reticulocytes, the diversity of relevant substrates suggests that other, perhaps undiscovered, E2 enzymes also participate in enhanced degradation. Among the proteins that are degraded more rapidly after the onset of differentiation are many enzymes and structural proteins (1), as well as apparent regulators of erythroid differentiation such as c-*myc* (48) and cyclin-dependent kinase 4 (49).

Reticulocytes have a high capacity for ubiquitin-dependent proteolysis (50, 51). In contrast, lysosomes, which contribute significantly to total protein turnover in most cell types (52), appear absent from reticulocytes (1). The total concentration
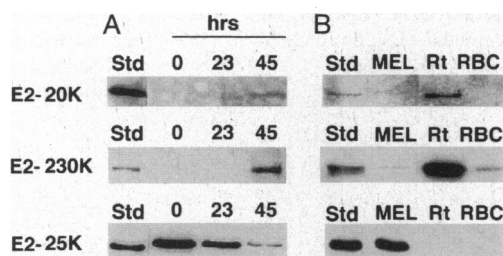

FIG. 4. Regulation of E2-20K and E2-230K protein levels during erythroid differentiation. The first lane of each panel is a purified quantitative standard (Std). (*A*) E2 induction during *in vitro* differentiation of FVA cells. At time zero, Epo was added to freshly isolated FVA cells. Cells were extracted at specific time points, as indicated. Analysis of Nonidet P-40 extracts from 10^6 (E2-20K, E2-230K) or 10^5 (E2-25K) FVA cells. Standards: 50 fmol of E2-20K, 20 fmol of E2-230K, and 150 fmol of E2-25K. (*B*) Induction of E2-20K and E2-230K is transient. MEL, undifferentiated MEL cells (a proerythroblast-like cell line); Rt, reticulocytes; RBC, erythrocytes. Each panel shows E2 levels in a fixed number of cells. Because reticulocytes are significantly smaller than their precursors, the intracellular concentrations of E2-20K and E2-230K in reticulocytes exceed those of MEL cells to a greater extent than shown, and the concentration of E2-25K falls to a lesser extent. Analysis of Nonidet P-40 extracts (MEL) or soluble protein (Rt, RBC) from 3×10^6 cells (E2-20K, E2-230K) or 10^5 cells (E2-25K). Standards: 50 fmol of E2-20K, 10 fmol of E2-230K, and 100 fmol E2-25K.

of ubiquitin is 5-fold higher in reticulocytes than in various cell lines and intact tissues (28). This result may be due, in part, to a significant induction of the polyubiquitin gene (UbB) (53) in differentiating erythroblasts (Fig. 3*A*). Moreover, 83% of ubiquitin is found in the conjugated form in reticulocytes, as opposed to 52% in MEL cells, a cell line similar to reticulocyte precursors (28). These observations suggest that ubiquitination is involved in degradation of many proteins during erythroid differentiation. Consistent with this view, the *in vitro* degradation of hexokinase (54) and reticulocyte mitochondrial proteins (55), which is associated with terminal differentiation, appears ubiquitin-dependent in reticulocyte lysates. Our results show a strong induction of two ubiquitin-conjugating enzymes in differentiating erythroid cells and suggest that the enhanced degradation observed in reticulocytes is driven not simply by increased availability of substrates for ubiquitination but also by qualitative alterations of the conjugation machinery itself. Induction of ubiquitin-conjugating enzymes could provide a simple mechanism for selective degradation of previously stable proteins inherited from progenitor cells.

The global reorganization of the erythroid cell can be viewed as a model for a variety of terminal differentiation processes. For example, the transformation of epithelial cells into lens fibers similarly involves enucleation, elimination of cytoplasmic organelles, conversion to glycolytic metabolism, and expression of a small number of specialized proteins (56, 57). Differentiation of lens fibers and erythroid cells, as well as less radical differentiation programs, entails broad alterations in the complement of cellular proteins, which generally depend on transcriptional regulation. Our results suggest that differentiation mechanisms can involve changes not only in gene expression but also in the specificity of the protein-degradation apparatus.

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