Retinoylation of Vimentin in the Human Myeloid Leukemia Cell Line HL60*

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Retinoylation (retinoic acid acylation) is a posttranslational modification of proteins occurring in many eukaryotic cell lines. The widespread occurrence of retinoylation suggests that it may play a role in many effects of retinoic acid (RA) on cells. The regulatory subunits of cyclic AMP-dependent protein kinase are retinoylated in the human myeloid leukemia cell line HL60 (Takahashi, N., Liapi, C., Anderson, W. B., and Breitman, T. R. (1991) Arch. Biochem. Biophys. 290, 293–302), and cytokeratins are retinoylated in normal human keratinocytes (Takahashi, N., Jetten, A. M., and Breitman, T. R. (1991) Biochem. Biophys. Res. Commun. 180, 393–400). We show, in this study, that the intermediate filament protein vimentin is retinoylated in HL60 cells during a 24-h exposure to 100 nM [3H]RA. We found that a retinoylated protein of Mr 55,000 coeluted on anion exchange chromatography and comigrated on either one- or two-dimensional polyacrylamide gel electrophoresis with a protein that also was stained on immunoblots with an anti-vimentin antibody. About 50% of the [3H]RA was released from this Mr 55,000 retinoylated protein after hydrolysis with either NH2OH (1 M, pH 10) or CH3OH, 0.1 M KOH. These results indicated that a large fraction of the RA was bound to vimentin by an ester bond. Both the Mr 55,000 retinoylated protein and immunoreactive vimentin were associated with cell nuclei isolated by two procedures. They were detached during exposure to a nonionic detergent buffer, suggesting that they are bound to the nuclear envelope. These results indicate that retinoylation is a new modification of vimentin that may be an early event in RA-induced differentiation of HL60 cells.

RA* induces terminal differentiation of the human acute myeloid leukemia cell line HL60 to cells with many functional and morphological characteristics of mature granulocytes (1). RA induces the differentiation in vitro of cells from patients with acute promyelocytic leukemia (2–5) and, as a sole agent, induces complete remission of patients with acute promyelocytic leukemia (6–10).

One mechanism for the differentiation-inducing activity of RA in a variety of cell types involves RA nuclear receptors (RARs) (11–18). These receptors are members of the steroid/thyroid nuclear receptor multigene family (15) and have specific high affinity binding sites for RA and some of its metabolites. Until recently, it was generally accepted that the actions of RA in development and cell differentiation are mediated by one or more of the nuclear receptors. These receptors directly activate transcription of their target genes by binding to specific DNA sequences. Direct support for a role of the nuclear receptors in differentiation is limited. RARs can mediate RA-induced differentiation of HL60 cells (17), and RARs is involved in RA-induced differentiation of P19 embryonal cells (18). In contrast, the RARs cannot be involved in the RA-induced differentiation of F9 embryonal carcinoma cells (19), and a correlation is not always seen between the biological potency of retinoids and their affinity for a RAR (20, 21).

Retinoylation may be another mechanism for the effect of RA on cells. We reported that in growing HL60 cells, RA binds via a covalent bond to preformed protein (22). The extent of this retinoylation depends on the initial concentration of RA in a saturable manner. Furthermore, the concentration-effect relationship for RA-induced differentiation and for retinoylation are similar (22, 23).

Retinoylation of proteins occurs in human myeloid leukemia cells in primary culture (24) and in cell lines that respond to RA, including the human breast tumor cell line MCF-7 (25, 26), mouse embryonal carcinoma cells, normal canine kidney epithelial cells (23), and normal human keratinocytes (27). Our findings of different retinoylated proteins in various cell types are consistent with the pleiotropic effects of RA.

The widespread occurrence of retinoylation suggests that it may play a role in many effects of RA. The identification of retinoylated proteins is one approach to an understanding of the function of retinoylation. We found that the regulatory subunits of cyclic AMP-dependent protein kinase types I and II are retinoylated in both HL60 (28) and MCF-7 cells (26) and that cytokeratins are retinoylated in normal human keratinocytes (27). How retinoylation affects the function of these proteins is under investigation in our laboratory.

In our initial study, we noted that a protein of Mr ~55,000 and pi ~5 is the most prominent retinoylated protein in HL60 cells (22). This protein comigrated on two-dimensional PAGE with a Coomasie Blue-stained protein. This observation prompted us to examine whether a structural protein is retinoylated. Here, we present evidence that the intermediate filament protein vimentin is retinoylated in HL60 cells.

MATERIALS AND METHODS

Cells—In this study, we used the human myeloid leukemia cell lines HL60 (29), HL60/MRI (30), a mutant that is more sensitive to RA than HL60 cells, and HL60/RA-res (31), a mutant that is resistant to differentiation by RA. Cells were maintained in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Inc.). Cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO2 in air and subcultured every week. We estimated cell number on an electric particle counter (Coulter Electronics, Hialeah, FL) and viability by trypan blue dye exclusion.

Incorporation of RA into HL60 Proteins—Exponentially growing cells were harvested by centrifugation and resuspended at a concentration of
2 x 10^9 cells/ml in serum-free medium consisting of RPMI 1640 supplemented with 10 mM HEPES pH 7.3, 5 μg/ml insulin, and 5 μg/ml transferrin (32). Unlabeled RA and radioactive RA ([11,12-3H], 40-60 Ci/ mmol (1 Ci = 3.7 x 10^10 Bq), DuPont NEN) dissolved in absolute ethanol were diluted into the growth medium. The final concentration of ethanol was no higher than 0.1%. After incubation for 24 h, cells were harvested by centrifugation (200 x g, 5 min) and washed with phosphate-buffered saline containing 600 μg of bovine serum albumin (essentially fatty acid-free, Sigma/ml).

**Cell Fractionation**—Cells (2 x 10^9/ml, 100-200 ml) were harvested by centrifugation (200 x g, 5 min). Nuclei were isolated at 4 °C by a detergent method (33). Briefly, cells were washed three times with PCS phosphate-buffered saline containing 600 pg of bovine serum albumin (essential fatty acid-free, Sigma/ml). The suspension was centrifuged at 200 x g for 10 min. The supernatant fraction was then centrifuged at 10,000 x g for 10 min to obtain a crude nuclear fraction. The crude nuclear fraction was suspended in 2 ml of Buffer A containing 1% Triton X-100, 10 mM KC1, 0.4 mg/ml aprotinin, 0.2 mg/ml leupeptin, and 15 μM 4-amidinophenylmethanesulfonfyl fluoride (Boehringer Mannheim). The resuspended cells were then disrupted with a Dounce homogenizer. The homogenate was centrifuged at 800 x g for 10 min to obtain a crude nuclear fraction. The crude nuclear fraction was suspended in 2 ml of Buffer A containing 1% Triton X-100, 10 mM KC1, 0.4 mg/ml aprotinin, 0.2 mg/ml leupeptin, and 15 μM 4-amidinophenylmethanesulfonfyl fluoride (Boehringer Mannheim). The supernatant fraction was removed carefully, and the pellet was resuspended in a small volume of PCS and treated as above. The pellet contained purified nuclei as judged by phase microscopy. In some experiments, contact of the cells with PCS buffer was extended from 10 to 20, 45, or 70 min.

**Chromatographic Separation of Retinoylated Nuclear Proteins of HL60 Cells**—HL60 cells (2 x 10^9/ml) were grown for 24 h in serum-free medium containing 100 nM [3H]RA. The following procedures were then performed at 0-4 °C. Cells (2 x 10^9) were harvested by centrifugation, washed with phosphate-buffered saline, and suspended in 1.6 ml of Buffer A (20 mM Tris-HCl buffer, pH 7.5, 0.25 mM sucrose, 2 mM MgCl2, 1 mM CaCl2, 10 mM KC1, 0.4 mg/ml aprotinin, 0.2 mg/ml leupeptin, and 15 μM 4-amidinophenylmethanesulfonfyl fluoride (Boehringer Mannheim)). The resuspended cells were then disrupted with a Dounce homogenizer. The homogenate was centrifuged at 800 x g for 10 min to obtain a crude nuclear fraction. The crude nuclear fraction was suspended in 2 ml of Buffer A containing 1% Triton X-100, 10 mM KC1, 0.4 mg/ml aprotinin, 0.2 mg/ml leupeptin, and 15 μM 4-amidinophenylmethanesulfonfyl fluoride (Boehringer Mannheim). The supernatant fraction was then centrifuged at 100,000 x g for 10 min. The supernatant fraction was then centrifuged at 100,000 x g for 1 h to yield a detergent-solubilized crude nuclear fraction. The detergent-solubilized crude nuclear fraction (2 ml containing about 18 mg of protein) was applied to a Mono Q column (5 x 50 mm, Pharmacia LKB Biotechnology, Inc.) equilibrated with Buffer B (20 mM potassium phosphate buffer (pH 6.8), 1 mM EDTA, and 2 mM 2-mercaptoethanol). The column was washed with 5 ml of Buffer B and then eluted with a gradient of 0-0.5 mM NaCl in Buffer B. Fractions of 1 ml were collected at a flow rate of 1.4 ml/min.

The amount of [3H]-labeled retinoylated protein was determined in each 100-μl portion of each fraction by the Bligh-Dyer procedure (34). After extraction, each residue was dried and dissolved in 0.2 ml of 10% SDS, and the radioactivity was measured in a liquid scintillation spectrometer. A 30-μl portion of some fractions was extracted by the Bligh-Dyer procedure and analyzed by SDS-PAGE and immunoblotting.

**One- and Two-dimensional PAGE**—Cells were extracted by the Bligh-Dyer (34) procedure. The delipidated residue was collected by centrifugation at 10,000 x g for 5 min in a microcentrifuge and then dried in a centrifugal vacuum device (Savant). Proteins were separated in a slab gel apparatus using the discontinuous system described by Laemmli (35). The dried residues were dissolved in sample buffer containing 0.25% 2-mercaptoethanol and heated at 100 °C for 5 min. Electrophoresis was performed on 10% gels (1.5 mm thick, NOVEX) at a constant voltage of 125 V/gel. Two-dimensional PAGE was according to O'Farrell (36). The proteins were separated in the first dimension by two-dimensional PAGE and then transferred after one- or two-dimensional PAGE to a polyvinyldene difluoride membrane (Millipore Corp.) using an electroblotter (NOVEX). The activities of antigens with monoclonal antibodies for vimentin (catalog no. V 5255, Sigma), desmin (catalog no. D 1033, Sigma), α-tubulin (catalog no. T 9026, Sigma), and β-tubulin (catalog no. T 4026, Sigma) were visualized with the Protein blot AP system (Promega Corp.) according to the manufacturer's instructions.

**Stability of Bound RA**—The chemical stability of the [3H]RA bound to delipidated protein was assessed by hydrolysis with 1 N NH4OH, pH 10, at 23 °C for 4 h or with 0.1 N KOH in CH3OH for 2 h at 20 °C under N2 gas as described (22).

**RESULTS**

Two-dimensional PAGE Patterns of Retinoylated and Coomassie Blue-stained Proteins—The major retinoylated protein in HL60 cells is a Mr ~55,000 acidic protein that comigrates with a Coomassie Blue-stained protein on two-dimensional PAGE (22). Compared with HL60 cells, the level of total retinoylated protein is about 5-fold greater in HL60/MRI cells, a mutant that is more sensitive to RA than HL60 cells, and about the same in HL60/RA-res cells, a mutant that is resistant to differentiation by RA (23). Furthermore, the level of the major retinoylated protein reflects the level of total retinoylated protein in each cell line (23). The major retinoylated protein of either HL60, HL60/MRI, or HL60/RA-res cells comigrated with a protein stained by Coomassie Blue (Fig. 1, protein 1). Only HL60/RA-res cells had a high level of protein 2, another retinoylated protein.
Anion Exchange Chromatography—In HL60 cells, most of the retinoylated protein were harvested by centrifugation, nuclei were isolated, and nuclear proteins were chromatographed on a Mono Q column as described under “Materials and Methods.” In panel A, the values for retinoylated protein (O) are the amounts present in each 1-ml column fraction. The dotted line shows the concentration of NaCl. Two portions of fraction 31 were separated by two-dimensional PAGE. One gel was stained with Coomassie Brilliant Blue (panel B) and then exposed to the film for 90 days (panel D). The proteins in the other gel were electrophobted and immunostained for vimentin (panel C).

Oyated protein (23) (Fig. 1). This protein had the same Mγ as a lower pl than protein 1 and comigrated with a Coomassie Blue-stained protein (Fig. 1). These results suggested that protein 1 could be an abundant cellular protein and that protein 2 could be its isoform.

A high level of the nonretinoylated protein 4 was seen only in HL60/RA-res cells (Fig. 1). This protein had about the same Mγ as the nonretinoylated protein 3, which we saw in the three cell lines. It is likely that proteins 3 and 4 are tubulins, based on mobilities on two-dimensional PAGE and positive immunostaining with anti-tubulin antibodies that did not stain protein 1 (data not shown).

The comigration of the major retinoylated protein (Fig. 1, protein 1) with a protein stained by the relatively insensitive Coomassie Blue reagent led us to examine whether it was the intermediate filament protein vimentin that has similar mobilities on two-dimensional PAGE (37–41).

Coelution of Vimentin and the Major Retinoylated Protein on Anion Exchange Chromatography—In HL60 cells, most of the cellular retinoylated protein is associated with nuclei isolated from cells disrupted either with Triton N-101 (22) or with a Dounce homogenizer (28). Proteins from nuclei isolated by the latter procedure were separated by anion exchange chromatography (Fig. 2A). We saw at least four peaks of retinoylated protein. The retinoylated proteins eluting at or below 0.3 M NaCl are the cAMP-binding regulatory subunits of types I and II cAMP-dependent protein kinase (28). Most of the retinoylated protein eluted at 0.4 M NaCl in a peak containing about 70% of the total radioactivity shown in the chromatogram. We separated the protein in fraction 31 (Fig. 2A) by two-dimensional PAGE. As shown in Fig. 2, B–D, a Coomassie Blue-stained protein, an anti-vimentin antibody reacting protein, and a retinoylated protein all had the same mobility on two-dimensional PAGE. We did not see a reaction with any protein when anti-α-tubulin monoclonal antibody was substituted for the anti-vimentin monoclonal antibody in the immunoblot analysis.

The chemical stability of the [3H]RA bound to protein in fraction 31 also was assessed. About 44% of the radioactivity was released from the protein in fraction 31 after treatment either with 1 M NH₄OH, pH 10, at 23 °C for 4 h or with 0.1 N KOH in CH₃OH for 2 h at 20 °C under N₂ gas. We showed previously that methyl [3H]retinoate is the major product of hydrolysis with CH₃OH:KOH of total retinoylated HL60 protein (22). Thus, about 50% of the RA bound covalently to protein in fraction 31 may be in ester (O-ester or thioster) linkage. The remainder may be in thioether, O-ether, amide, or another linkage (42).

Intracellular Distribution of the Major Retinoylated Protein and Vimentin—We observed previously (43) that the major retinoylated protein was released from an HL60 nuclear preparation by an extended exposure to Triton N-101. As shown in Fig. 3A, covalently bound RA accumulated in the low speed supernatant fraction after HL60 cells were suspended in the Triton N-101-containing buffer. About 80% of the covalently bound RA was associated with the nuclear pellet following a 10-min exposure to Triton N-101. After an additional 10-min exposure (total exposure time of 20 min) about 55% of the covalently bound RA was in the pellet fraction. This value did not change markedly for a further 50 min though the prolonged exposure to Triton N-101 was associated with a decreased nuclear diameter and the appearance of a gel-like nuclear envelope as seen under light microscopy.

We further characterized the [3H]retinoylated protein of cells exposed to Triton N-101 for 20 min (Fig. 3). Proteins in the supernatant and pellet fractions were separated by one-dimensional PAGE. A protein band of Mγ, 55,000 from both the Triton N-101 supernatant (Fig. 3B) and pellet (data not shown) fractions stained with anti-vimentin antibody. A protein band of Mγ, 55,000 from the supernatant fraction was labeled by [3H]RA (Fig. 3C). The one-dimensional PAGE fluorogram of the retinoylated proteins from the Triton N-101 pellet fraction did not give a sharp radioactive band at Mγ, 55,000, apparently because
the other retinylated nuclear proteins (Fig. 2A) were concentrated in the Triton N-101 pellet. Therefore, proteins in the Triton N-101 pellet fraction were separated by two-dimensional PAGE, and we saw a protein of Mr. ~55,000 (Fig. 3F, arrow) that had the same mobility as protein 1 (Fig. 1) and the protein in fraction 31 (Fig. 2, B–D).

The specificity of the vimentin antibody reaction was supported by our findings that a Mr. 55,000 protein in either the supernatant or pellet fractions was not stained when anti-desmin monoclonal antibody was substituted for the anti-vimentin monoclonal antibody (Fig. 3, D and E). The anti-desmin antibody stained primarily at Mr. 52,000 protein and, at a lower intensity, a Mr. 50,000 protein in the pellet fraction (Fig. 3E). No proteins were stained in the supernatant fraction (Fig. 3D).

**DISCUSSION**

We have shown, in this study, that vimentin may be retinoylated in HL60 cells. Our evidence is the comigration on anion exchange chromatography and on one- or two-dimensional PAGE of both a retinylated protein and a protein stained by anti-vimentin antibody or Coomassie Brilliant Blue R-250 (Figs. 1–3). In addition, a retinylated protein and a protein reacting with anti-vimentin antibody, both with Mr. values of 55,000, are co-extracted into a Triton N-101-soluble fraction of intact cells (Fig. 3). We previously showed that most HL60 cellular retinylated protein is associated with nuclei prepared by a 10-min exposure of intact cells to Triton N-101 (43). Retinylated protein extracted during an additional exposure of purified nuclei to Triton N-101 binds to DNA-cellulose and is eluted with NaCl (43). Vimentin is also a DNA-binding protein with similar properties (44). This is additional evidence that the major retinylated protein in HL60 cells may be vimentin.

By immunofluorescence microscopy, the level of vimentin in undifferentiated HL60 cells is very low and is seen as a diffuse nonfilamentous form (37, 45–47). During differentiation along the monocye/macrophage lineage by 12-O-tetradecanoylphorbol-13-acetate, HL60 cells develop a vimentin network similar to that seen in normal monocytes/macrophages (37, 46, 47). In contrast, differentiation of HL60 cells along the granulocytic pathway by either RA or dimethyl sulfoxide results either in no appreciable change in the distribution of vimentin (46) or in the appearance of a filamentous network of vimentin close to the nucleus (45, 47), which is similar to that seen in normal human mature granulocytes (46).

The intracellular location of vimentin in HL60 cells is not clearly defined. This information may be essential in evaluating the role of retinoylation of this protein. Vimentin is seen primarily in the cytosol of many cell types using fluorescence microscopy (37–39, 48–57). However, vimentin copurifies with nuclei isolated by various cell fractionation procedures (58–61). Our results agree with these latter results. We found that most of either the Mr. 55,000 retinylated protein or vimentin is isolated with nuclei prepared from cells disrupted either with Triton N-101 and a low speed centrifugation (Fig. 3) (22) or by homogenization followed by differential centrifugation (Fig. 2) (28). The association of vimentin with isolated nuclei from some cell types has been ascribed to cytoplasmic contamination (58, 59, 61, 62). The removal of vimentin and the retinylated protein from a cell nuclear pellet by exposure to Triton N-101 (Fig. 3) suggests that vimentin is attached to the periphery of the nuclear envelope. This would be consistent with findings that vimentin binds strongly to lamin B in the lamina-pore complex (63, 64). Thus, the association of vimentin with the cell nucleus seen after subfractionation of disrupted cells may represent the status in the intact cell.

Changes in the intracellular distribution, the amount, or the rate of new synthesis for vimentin is seen in HL60 cells induced to differentiate by RA to granulocytoid cells or by 12-O-tetradecanoylphorbol-13-acetate to monocytoid/macrophage-like cells (37, 45, 51). Leung et al. (45) saw no significant difference in the levels of vimentin protein, quantitated by flow cytometry, in HL60 cells grown for 24 h with 1 μM RA. At day 4, Leung et al. (45) saw increases in vimentin of about 1.3-fold in cells grown with 100 nM RA and about 1.7-fold in cells grown with 1 μM RA. Under the latter conditions, the organization of vimentin in the cell changed from one without filamentous structures into one with a filamentous network.

Vimentin is not the only intermediate filament protein that is retinoylated. We showed previously (27) that cytokeratins are retinylated in undifferentiated and differentiated normal human keratinocytes. We noted, in that study, the retinoylation of a protein that migrates identically to protein 1 (Fig. 1) (27). During RA-mediated differentiation of keratinocytes, the relative retinoylation of protein 1 decreases, while the relative retinoylation of the cytokeratins increases. This is the expected pattern for changes in the levels of vimentin and the cytokeratins in differentiating keratinocytes (65, 66).

We also saw evidence for retinoylation of vimentin in the human breast tumor cell lines MCF-7 and MCF-7/AdR (25, 26). We showed that both cell lines have a low level of a retinylated protein that migrates identically to protein 1 (Fig. 1). The level of this retinylated protein is lower in MCF-7 cells than in MCF-7/AdR cells (26). These results are consistent with reports that the levels of vimentin are very low or undetectable in MCF-7 cells (38, 54, 67–69) and detectable in MCF-7/AdR cells (68, 69). There is an inverse relationship between the levels of vimentin and cytokeratins in breast tumor cell lines (38), and vimentin expression in human breast tumor tissue is associated with an increased metastatic phenotype (70, 71). In contrast to our findings with keratinocytes (27), we did not see retinoylation of the cytokeratins in either MCF-7 or MCF-7/AdR cells (26).

Although our results show that vimentin is covalently modified by RA, the possible role of this retinoylation remains to be elucidated. We calculate that <1% of the vimentin is retinoylated in HL60 cells grown in the presence of 100 nM [3H]RA for 24 h. We previously showed that the level of retinoylation of HL60 proteins was 5-fold higher after exposure to 1 μM RA for 24 h and reached a constant level/cell after growth for 48 h in the presence of 100 nM RA. Thus, even at pharmacologic levels of RA, a low percentage of the total vimentin may be retinoylated. At one extreme, it is possible that all (100%) of the vimentin in <1% of the cells is retinoylated. We have shown previously that retinoylation, like palmitoylation and phosphorylation, is a dynamic process occurring on preformed protein (43). If the retinoylation process has a shorter half-life than vimentin protein, then, at the 24-h time point, we measured only a fraction of the total vimentin molecules that were retinoylated from zero time.

The possibility that retinylated vimentin is distributed differently in the cell or has changes in physical properties remains to be addressed in more detail. From our data, it appears that retinylated and nonretinylated vimentins share properties such as comigration on two-dimensional PAGE (Fig. 1), colocalization on an anion exchange column (Fig. 2), colocalization in the cell (Fig. 3), and binding to DNA (43). Retinoylation is at least the second posttranslational modification known for vimentin. The phosphorylation of vimentin is well documented (72–74) and has been implicated as playing a critical role in cytoplasmic organization and cell cycle progression. The disassembly of vimentin following phosphorylation is believed to be the basis for structural changes in the cell. Vimentin is phosphorylated in intact cells and in cell-free systems by several protein kinases, including protein kinase A,
protein kinase C, and p34cdc2 (48, 73-80). Phosphorylated forms of vimentin have more acidic pI values on two-dimensional PAGE (48, 74-76, 81, 82). It is likely that protein 2, present at high concentrations in HL60/RA-res cells (Fig. 1), is a phosphorylated form of vimentin. Protein 2 also is seen in HL60 res cells (83), another RA-resistant mutant of HL60 cells.² The role, if any, of protein 2 in RA resistance remains to be determined. One possibility is that it may interfere with a function of vimentin, e.g., the intracellular movement of low density lipoprotein-derived cholesterol from the lysosome to the site of esterification (54).

In this study, we focused on determining whether vimentin in HL60 cells, to increase the level of retinoylation by either increasing the concentration of RA or increasing the time of exposure to RA, to determine the half-life of the covalently bound RA moiety compared with the half-life of vimentin protein, and to identify the RA moiety and the site(s) at which it is covalently bound to vimentin. This information should aid in elucidating the role of retinoylation in differentiation and throw new light on the function of vimentin.

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REFERENCES


² N. Takahashi and T. R. Breitman, unpublished results.