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Proteins in Human Myeloid Leukemia Cell Line HL60 Reacting with Retinoic Acid Monoclonal Antibodies

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The vitamin A derivative, retinoic acid (RA) has various biological effects in mammalian cells and tissues. It is well known that RA induces differentiation of leukemia cells and inhibits cell growth. There are two pathways for RA action; one *via* RA nuclear receptors (RARs), and one *via* acylation of proteins by RA (retinoylation). However, an understanding of which actions of RA occur *via* RARs and which occur *via* retinoylation is lacking. Thus, we undertook the examination of HL60 proteins using anti-RA monoclonal antibodies (ARMAs). These ARMAs showed specific binding to proteins in a saturable manner depending on protein and antibody concentration. Proteins eluted by Mono Q anion exchange chromatography and separated using two-dimensional polyacrylamide gel electrophoresis were detected by ARMAs. One of these ARMA-bound proteins in HL60 cells was identified as α-actinin. These results indicate that retinoylated proteins in HL60 cells can be recognized by ARMAs and that α-actinin modified by RA may play a significant role in RA-induced differentiation, including the promotion of cytomorphology changes.

Key words: HL60 cells, retinoic acid, retinoylated protein, retinoylation, α-actinin.

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Retinoic acid (RA), a carboxylic acid derivative of vitamin-A (retinol), plays many roles in mammalian cells and tissues. Based on nutritional studies with vitamin A-deficient animals, RA supports growth in animals and maintains epithelial tissues and bone, but does not function in vision and mammalian reproduction. More recently, studies with mice lacking specific RA nuclear receptors (RARs) show that RA plays a crucial role in spermatogenesis and reproduction (*1*). Underscoring the importance of RA is its potent induction of differentiation of some cell types and its utility in the treatment of patients with various malignancies, especially acute promylocytic leukaemia (*2*). In light of the reported biological effects of RA, clarifying mechanisms of RA action is of critical interest.

One mechanism for the activity of RA in a variety of cell types, involves RA nuclear receptors (RARs and retinoid X receptors) (*1*, *3–6*). The action of RA in development and cell differentiation is mediated by these receptors, which directly activate or repress transcription of their target genes by binding to specific DNA sequences. However, some effects of RA are nongenomic (*7–11*). Therefore it is possible that other mechanisms, in addition to RA binding to RA receptors, may be involved in the biologic effects of RA.

Retinoylation (acylation of proteins by RA) is another mechanism by which RA may act on cells (*12–18*). Retinoylation is a form of protein postranslational modification that occurs in a variety of cell types *in vitro* (*12–18*) and in tissues *in vivo* (*19*). Metabolic pathways for retinoylation employ the formation of retinoyl-CoA intermediates (*20*, *21*) with subsequent transfer of the retinoyl moiety to proteins (*22*). The ATP-dependent generation of retinoyl-CoA occurs in cells and tissues and may play a significant physiological role in the actions of RA mediated by retinoylation (*20*, *21*).

RA induces terminal differentiation of human acute myeloid leukemia HL60 cells to cells having morphologic characteristics of mature granulocytes. In growing HL60 cultures, RA is linked *via* a thioester bond to proteins, and the extent of retinoylation is dependent in a saturable manner on the initial concentration of RA (*13*, *16*). Dose-response curves for RA-induced differentiation and retinoylation are similar, with positive correlations (*13*, *16*). It is also known that the regulatory subunits of type I and type II cyclic AMP-dependent protein kinase (PKA) (*14*) and vimentin (*17*) in HL60 cells are retinoylated. It is becoming increasingly evident that retinoylation may be a true physiological function of RA.

Previous studies have shown that anti-RA monoclonal antibodies (ARMAs) (*23*, *24*) exhibit specificity against RA (*23*, *24*). These ARMAs have been used in studies of the distribution of retinoids in the chick lim bud (*23*), in early quail embryos (*25*), in human premalignant oral lesions (*26*), and they have been utilized to neutralize endogenous RA (*27*). However, ARMAs have not been used to examine the components responsible for RA-induced cell differentiation. In this study, we demonstrate the ability of ARMAs to bind to proteins in RA-treated HL60 cells, and we identify a novel retinoylated protein that may participate in RA-induced differentiation.

METHODS

Cells—Human myeloid leukemia HL60 cells were maintained in serum-containing medium (GIBCO) consisting

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of RPMI 1640 supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), pH 7.3 and 10% (v/v) fetal bovine serum (FBS)(GIBCO). Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cell number was determined with an electronic particle counter (Coulter Electronics) and cell viability was assessed by trypan blue dye exclusion.

Incorporation of RA into HL60 proteins—Exponentially growing cells were harvested by centrifugation and resuspended at a concentration of 2×10^6 cells ml^{-1} in serum-free medium consisting of RPMI 1640 supplemented with 10 mM HEPES, 5 µg of insulin ml^{-1} and 5 µg of transferrin ml^{-1}. Retinoic acid was dissolved in absolute ethanol and diluted into the growth medium. The final concentration of ethanol was no higher than 0.1%. Cells incubated with 100 nM RA for 24 h, were harvested by centrifugation ($200 \times g$, 10 min) and washed with PBS. In order to remove free RA, cells incubated with RA were extracted five times with $CHCl_3 : CH_3OH : H_2O$ (1 : 2 : 0.8) using the Bligh–Dyer procedure (*28*) and centrifuged at $10{,}000 \times g$ for 5 min in a microcentrifuge. This extraction was repeated approximately five times. The resulting delipidated pellet was dried in a centrifugal vacuum device (Sakuma, Tokyo, Japan), and dissolved in sample buffer.

Two-dimensional polyacrylamide gel electrophoresis—Samples were prepared as follows: The delipidated residue resulting from 4×10^6 cells as described above was dissolved in isoelectric focussing buffer containing 9.5 M urea, 2% NP-40 and 2% ampholytes (pH 3.5~10, Amersham Biosciences Co.). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed according to O'Farrell (*29*). First isoelectric-focussing gels containing 2% ampholytes (pH 3.5 ~ 10) were run along one axis. Next, 10–20% gradient polyacrylamide gels were run along the second axis (Anatech, Tokyo, Japan). Prestained protein molecular weight markers were obtained from Bethesda Research Laboratory (GIBCO).

Preparation of subcellular fractions of HL60 cells treated with RA—Cells (2×10^6 cells ml^{-1}) were incubated with 100 nM RA and harvested by centrifugation ($200 \times g$, 10 min). Cells ($1.0 \sim 2.0 \times 10^9$ cells) were resuspended with 2 or 3 ml of Buffer A [10 mM Tris-HCl buffer (pH 7.5) and 0.005% (v/v) Tween 20], and homogenized using an Ultra-Turrax (IKA-Analysentechnik GmbH, Heitersheim, Germany). After centrifugation of homogenates ($100{,}000 \times g$, 60 min), the resulting supernatant was used as the soluble extract. The precipitated fraction was dissolved with Buffer A supplemented with 10% (w/v) 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate (CHAPS) and homogenized (Ultra-Turrax). After centrifugation ($100{,}000 \times g$, 60 min), the resulting supernatant was used as the insoluble extract. Subcellular fractions were divided into tubes and stored at −80°C so as not to freeze-thaw. Storage was no longer than 2 months prior to use.

Chromatographic separation of proteins in soluble and insoluble extracts of HL60 cells treated with RA—Soluble and insoluble extracts prepared as described above were applied to Mono Q columns (5×50 mm, Pharmacia LKB) equilibrated with Buffer B [20 mM potassium phosphate buffer (pH 6.8), 1 mM EDTA, and 2 mM 2-mercaptoethanol]. The columns were washed with 5 ml of Buffer B and then eluted with a gradient of 0 to 0.5 M NaCl in Buffer B. Fractions of 1 ml were collected at a flow rate of 0.5 ml min^{-1}.

Preparation of anti-RA monoclonal antibody—Hybridoma cells of anti-RA monoclonal antibodies (ARMAs) in serum-containing RPMI 1640 medium were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Anti-RA monoclonal antibodies were prepared as described previously (*23*), and the IgG fraction was purified using affinity column chromatography with protein G.

Detection of proteins by immunoblot analysis—Proteins were separated by 2D-PAGE as described above and one-dimensional polyacrylamide gel electrophoresis (1D-PAGE). 1D-PAGE was performed according to Laemmli's method (*30*). Briefly, samples were heated at 100°C for 5 ~ 10 min in solution [125 mM Tris-HCl buffer (pH 6.8), 4% (w/v) SDS, 40% glycerol, 10% (v/v) 2-mercaptoethanol and 0.005% (w/v) bromophenol blue]. Electrophoresis on 7%, 12.5% or 15% polyacrylamide gels (1.0 mm thick) was then performed. Proteins were separated by 1D- or 2D-PAGE, then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Co., USA) using an electroblotter (Bio Craft, Japan) at 50 mA overnight. After blocking for 1 h the membrane was incubated with the first antibody for 2 h and then the second antibody for 1 h at room temperature. The antigen–antibody reactivity was visualized by chemiluminescence staining using an ECL plus Western blotting starter Kit (Amersham Bioscience Co., USA) according to the manufacturer's instructions. Gels were stained using a silver staining II kit (Wako Pure Chemical Industries, LTD., Osaka, Japan).

Purification, digestion, separation, and amino acid sequencing of proteins—Proteins (400 µl) in fraction 16 of the insoluble extract (1 ml) were separated by 1D-PAGE (7%) and detected using ARMAs. The portion of gel corresponding to a single band detected by ARMAs was cut out and directly digested with lysyl endopeptidase (Wako Pure Chemical Industries, Ltd., Osaka, Japan), or V8 proteinase (Sigma, St. Louis, MO), under optimum conditions according to the manufacturer's instructions. The resulting peptides were extracted from the gel with 50% acetonitrile containing 5% trifluoroacetic acid, dried in a centrifugal vacuum (Sakuma, Tokyo, Japan), and dissolved in 0.1% trifluoroacetic acid. The resulting peptides were separated by a µ-blotter system (ABI 173A, PE Applied Biosystems, Foster City, CA, USA) using a linear gradient of 0 ~ 80% acetonitrile containing 0.1% trifluoroacetic acid with a flow rate of 5 ml min^{-1}. Absorbance was monitored at 210 nm. Peptide fragments were sequenced by automated Edman degradation using a PROCISETM 492 cLC Protein Sequencer (ABI 173A, PE Applied Biosystems).

RESULTS

Reactivity of ARMAs with HL60 protein—The specificity of ARMAs was first determined. HL60 cells treated with 100 nM RA were fractionated as soluble and insoluble extracts. Each fraction was applied to

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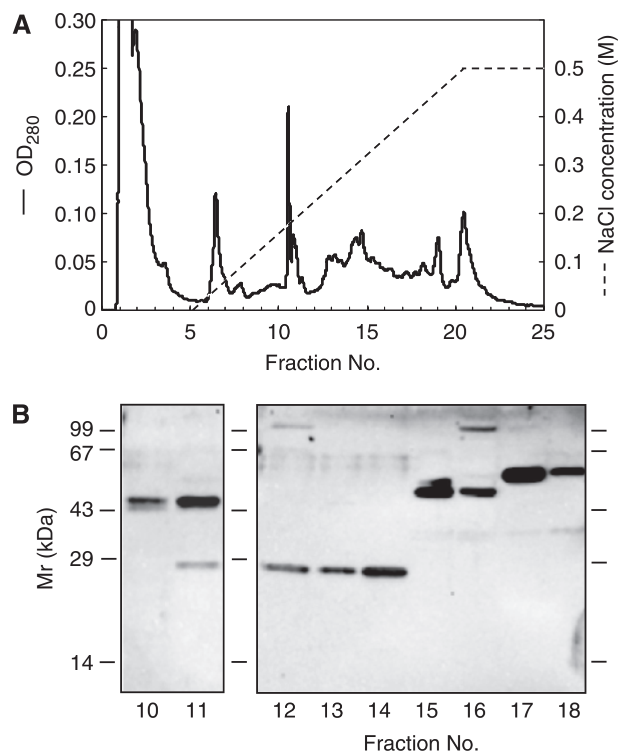

Fig. 1. **Anion-exchange chromatography of proteins in soluble extract of HL60 cells and immunoblotting with ARMAs.** HL60 cells grown in serum-free medium were harvested by centrifugation and homogenized. Soluble cell extract was chromatographed on a Mono Q column (A). Proteins (10 μl) in 1 ml-column fractions were separated by SDS-PAGE (12.5%) and analysed by immunoblotting with ARMAs (Dilution; 1/100) (B). Performed as described under "Methods".

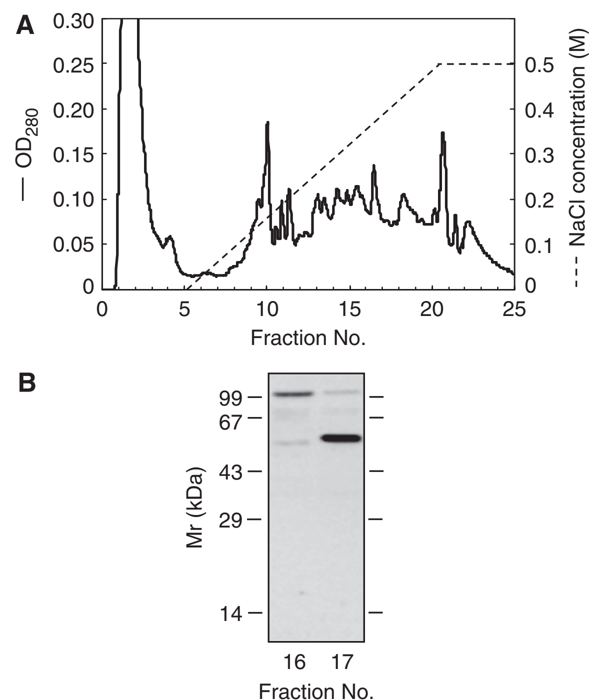

Fig. 2. **Anion-exchange chromatography of proteins in insoluble extract of HL60 cells and immunoblotting with ARMAs.** HL60 cells grown in serum-free medium were harvested by centrifugation and homogenized. Insoluble cell extract was chromatographed on a Mono Q column (A). Proteins (10 μl) in 1 ml-column fractions were separated by SDS-PAGE (12.5%) and analysed by immunoblotting with ARMAs (Dilution; 1/100) (B). Performed as described under "Methods".

anion-exchange chromatography columns (Mono Q), and proteins were eluted with a gradient of 0 to 0.5 M NaCl. The elution pattern of proteins from the soluble extract is shown in Fig. 1A. To detect proteins bound to ARMAs, immunoblot analysis was performed. Proteins reacting with ARMAs were observed in fractions 10 ~ 18. Fractions 10 ~ 11 and 11 ~ 14 contained ARMA-binding proteins with molecular weights of approximately 47 and 28 kDa, respectively (Fig. 1B). An ARMA-binding protein exhibiting a molecular weight of approximately 51 kDa eluted in fractions 15 and 16 at 0.3 ~ 0.34 M NaCl. A protein of approximately 100 kDa molecular weight was observed in fractions 16 and 17 (0.34 ~ 0.38 M NaCl) as well as an approximately 55 kDa molecular weight protein in fractions 17 and 18 (0.38 ~ 0.4 M NaCl). These were eluted sequentially (Fig. 1B). Figure 2A shows chromatograms of proteins in the insoluble extract. The elution pattern of proteins was distinct from that in Fig. 1A. The amounts of proteins in the insoluble extract were more than in the soluble extract. As shown in Fig. 2B, proteins bound to ARMAs are contained in fractions 16 and 17. The molecular weights of these proteins were approximately 100 kDa for fractions 16 and 17 (0.34 ~ 0.38 M NaCl), approximately 51 kDa for fraction 16 (0.34 M NaCl) and approximately 55 kDa for fraction 17 (0.38 M NaCl). These proteins were also seen in Fig. 1B.

Proteins in fraction 16 and 17 were examined by immunoblotting using various ARMA concentrations. Figure 3 shows 1D-PAGE patterns of proteins in the insoluble extract that bound to ARMAs. The protein in fraction 16 exhibiting a molecular weight of approximately 100 kDa was observed with all ARMA dilutions (a: 1/100, b: 1/1000, c: 1/10,000), while its density was reduced in the order of a, b and c and saturated in the order of c, b and a (Fig. 3A(a–c), upper band). Similar results were seen with the protein in fraction 17 exhibiting a molecular weight of approximately 55 kDa. This band disappeared with 1/10,000 dilution of ARMAs (Fig. 3A(c)). Next, when the protein concentration was changed, the density of bands decreased in parallel with the diminishing concentration of proteins (Fig. 3B(d–e)). In contrast, no proteins were detected, when ARMAs were pretreated with cold RA (data not shown). These results indicate that the detection of protein was dependent both on the ARMA content and protein in a saturable manner, suggesting that the ARMAs were specific for RA.

Anti-RA monoclonal antibodies can react with RA bound to proteins both non-covalently and covalently. Proteins in fractions eluted from Mono Q columns contain both forms of proteins. However, because the separation of HL60 proteins by 1D-PAGE containing detergents cleaves non-covalently bound RA, but not

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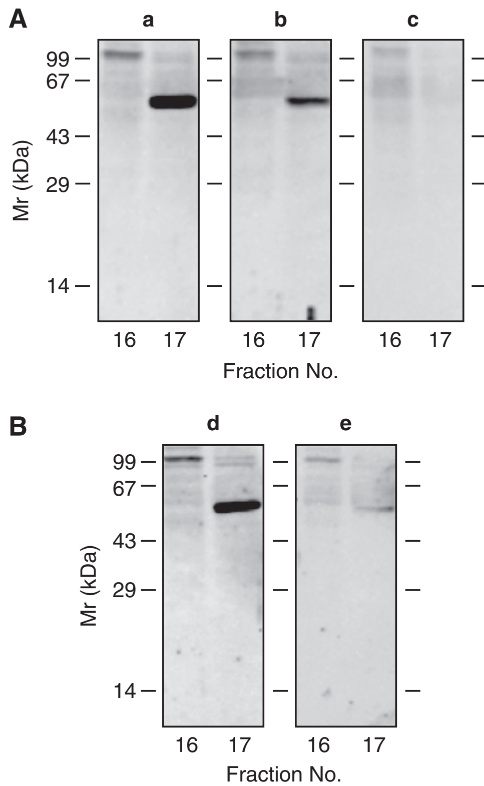

Fig. 3. **The specificity of ARMAs.** Proteins from insoluble extract (1 ml) in fractions 16 and 17 shown in Fig. 2 were separated by SDS-PAGE (12.5%) and analysed by immunoblotting with ARMAs as described under "Methods". (A) Protein: 10 µl. ARMA dilution: a, 1/100; b, 1/1,000; c, 1/10,000. (B) Protein: d, 10 µl; e, 5 µl. ARMA dilution: 1/100.

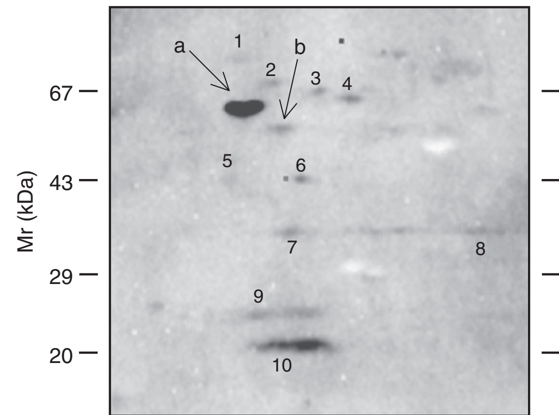

Fig. 4. **2D-PAGE showing retinoylated proteins detected by ARMAs.** HL60 cells (2×10^6 cells ml^{-1}) were grown for 24 h in serum-free medium containing 100 nM RA. Cells were harvested by centrifugation. After extraction by the Bligh-Dyer procedure, the proteins from 4×10^6 cells were separated by 2D-PAGE with 10–20% gradient SDS-polyacrylamide gel. The gel was analysed by immunoblotting with ARMAs (Dilution; 1/100) as described under "Methods".

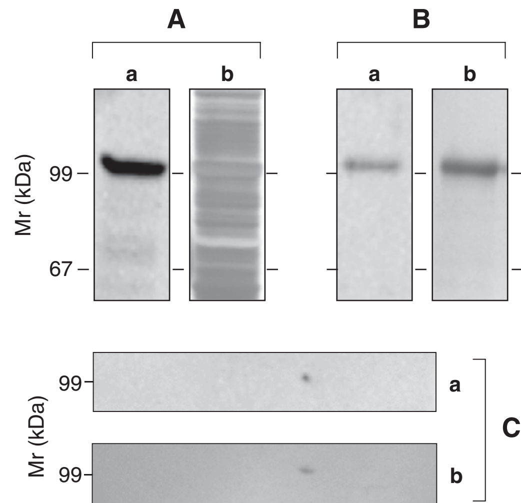

Fig. 5. **Purification of a retinoylated protein (p100^{RA}) in fraction 16 of insoluble extract.** Proteins in fraction 16 of insoluble extract shown in Fig. 2 (A) and a retinoylated protein purified from fraction 16 (p100^{RA}) of insoluble extract (B) (C) were separated by 1D-PAGE and 2D-PAGE (7%), and were analysed by immunoblotting with ARMAs (a) and silver protein staining (b) as described under "Methods".

covalently bound adduct, the proteins detected by immunoblotting with ARMAs have RA covalently linked to the proteins, and are therefore termed "retinoylated proteins".

2D-PAGE pattern of retinoylated proteins detected by ARMAs—Next it was determined whether proteins in total cells after removing free RA separated by 2D-PAGE could be detected by ARMAs. A 2D-PAGE pattern of proteins detected by ARMAs is shown in Fig. 4. The major protein (Arrow a), the minor proteins (Arrow b) and other proteins (proteins 1–10) were reacted with ARMAs. These results suggested that ARMAs could recognize retinoylated proteins separated by 2D-PAGE.

Identification of retinoylated protein detected by ARMAs—Retinoylated protein detected by ARMAs and contained in fraction 16, were eluted from a Mono Q column. Both soluble and insoluble extracts from HL60 cells containing protein exhibiting a molecular weight of approximately 100 kDa (p100^{RA}) were examined (Figs 1 and 2).

In order to purify the retinoylated protein p100^{RA}, proteins in fraction 16 of the insoluble extract were separated by 1D-PAGE (7%), and then analysed by immunoblotting with ARMAs or by silver staining. As shown in Fig. 5A, a single band reacted with ARMAs and this exhibited a molecular weight of approximately 100 kDa (Fig. 5A(a)). It should be noted that many protein bands were seen by silver staining (Fig. 5A(b)). After the band detected by ARMAs was cut out, a protein was extracted from the gel and isolated. The purified protein was separated by 1D-PAGE (7%) and immunoblotting was performed with ARMAs and silver staining. As shown in Fig. 5B, a single protein band at the same molecular weight was seen in both the immunoblot (Fig. 5B(a)) and silver staining gel (Fig. 5B(b)). Purified p100^{RA} was analysed by 2D-PAGE and a single spot of protein reacted with ARMAs (Fig. 5C(a)) and visualized by silver staining (Fig. 5C(b)).

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The purified retinoylated protein, p100[RA] could not be analysed by automated Edman degradation, indicating that the *N*-terminal end of the polypeptide chain was blocked by modification. Therefore, purified p100[RA] was directly digested with lysyl endopeptidase (peptide A and C), as well as V8 proteinase (peptide B) under optimum conditions. The resulting peptides were separated by a μ-blotter system. Peptide fragments were sequenced by a Protein Sequencer, and the internal amino acid sequences of the peptide fragments (peptides A, B and C) were analysed as shown in Table 1. When the amino acid sequences obtained from the three peptide fragments were retrieved from the database, p100[RA] was identified as the actin binding protein α-actinin. Figure 6 shows the amino acid sequence of α-actinin. The sequences of peptides A, B and C (Table 1) were matched to the amino acid sequences of α-actinin (shown in Fig. 6). These results indicate that α-actinin is modified by RA in HL60 cell during RA-induced differentiation.

Table 1. **Codon of peptide fragments of a retinoylated protein in fraction 16 of insoluble extract determined by protein microsequencing.**

Peptide fragment	Amino acid sequence
Peptide A	LMLLLEVISG
Peptide B	IKALIRKH
Peptide C	MEEIGRISIEMNGTLEDQLS

Amino acids are listed by their single letter designation.

DISCUSSION

In the current study, several retinoylated proteins in HL60 cells were detected by ARMAs, which specifically recognize RA bound to proteins both covalently and non-covalently (Figs 1–4). The identification of a retinoylated protein, α-actinin, an actin-binding protein was accomplished using this technique (Table 1, Figs 5–6).

Previously, we reported RA acylation (retinoylation) of proteins in mammalian cells (*15*, *16*). Using conventional methodology that relies on radioactive RA, retinoylated

1 MVDYHAANQS YQYGPSSAGN GAGGGGSMGD YMAQEDDWDR DLLLDPAWEK QQRKTFTAWC

61 NSHLRKAGTQ IENIDEDFRD GLK**LMLLLEV ISG**ERLPKPE RGKMRVHKIN NVNKALDFIA

peptide A

121 SKGVKLVSIG AEEIVDGNAK MTLGMIWTII LRFAIQDISV EETSAKEGLL LWCQRKTAPY

181 KNVNVQNFHI SWKDGLAFNA LIHRHRPELI EYDKLRKDDP VTNLNNAFEV AEKYLDIPKM

241 LDAEDIVNTA RPDEKAIMTY VSSFYHAFSG AQKAETAANR ICKVLAVNQE NEHLMEDYEK

301 LASDLLEWIR RTIPWLEDRV PQKTIQEMQQ KLEDFRDYRR VHKPPKVQEK CQLEINFNTL

361 QTKLRLSNRP AFMPSEGKMV SDINNGWQHL EQAEKGYEEW LLNEIRRLER LDHLAEKFRQ

421 KASIHEAWTD GKEAMLKHRD YETATLSD**IK ALIRKH**EAFE SDLAAHQDRV EQIAAIAQEL

peptide B

481 NELDYYDSHN VNTRCQKICD QWDALGSLTH SRREALEKTE KQLEAIDQLH LEYAKRAAPF

541 NNWMESAMED LQDMFIVHTI EEIEGLISAH DQFKSTLPDA DREREAILAI HKEAQRIAES

601 NHIKLSGSNP YTTVTPQIIN SKWEKVQQLV PKRDHALLEE QSKQQSNEHL RRQFASQANV

661 VGPWIQTK**ME EIGRISIEMN GTLEDQLS**HL KQYERSIVDY KPNLDLLEQQ HGLIQEALIF

peptide C

721 DNKHTNYTME HIRVGWEQLL TTIARTINEV ENQILTRDAK GISQEQMQEF RASFNHFDKD

781 HGGALGPEEF KACLISLGYD VENDRQQEAE FNRIMSLVDP NHSGLVTFQA FIDFMSRETT

841 DTDTADQVIA SFKVLAGDKN FITAEELRRE LPPDQAEYCI ARMAPYQGPD AVPGALDYKS

901 FSTALYGESD L

Fig. 6. **Amino acid sequence of α-actinin, a retinoylated protein (p100[RA]) in fraction 16 of insoluble extract determined by protein microsequencing.** The amino acid sequences of three fragments derived from p100[RA] determined by protein microsequensing (Table 1) are underlined and bold. Amino acids are listed by their single letter designation.

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proteins from HL60 cells exhibiting molecular weights of approximately 47 and 51 kDa separated by Mono Q column chromatography and 2D-PAGE, have been identified as RIα and RIIα, which are the cAMP-binding regulatory subunits of type I and II PKA, respectively (*14*, *31*). Additionally, a retinoylated protein exhibiting a molecular weight of approximately 55 kDa has been identified as vimentin (*17*). In the current study, retinoylated proteins eluted from Mono Q columns reacting with ARMAs (47 kDa protein in fractions 10∼11, 51 kDa protein in fractions 15∼16, and 55 kDa protein in fractions 17∼18, respectively; Fig. 1) were identical to retinoylated proteins detected by conventional methods (*14*, *18*). The proteins stained with ARMAs exhibiting molecular weights of approximately 47 kDa in fractions 10∼11, 51 kDa in fractions 15∼16, and 55 kDa in fractions 17∼18 were also coimmunostained with antibodies against RIα, RIIα and vimentin, respectively (data not shown). In addition, 2D-PAGE patterns of retinoylated proteins stained with ARMAs were similar to those labelled with [^{3}H]-RA (*14*, *18*). These results indicate that while both ARMAs and [^{3}H]-RA can be used to recognize retinoylated proteins in HL60 cells, the detection of retinoylated proteins by classical methods using [^{3}H]-RA requires significantly longer time and special instrumentation (*12–19*). Detection times on the order of several months were needed, even though enhancer solutions and enhancer screens were used. In contrast, ARMAs could detect retinoylated proteins in a few days without radioactive materials. This represents a major improvement in the detection of retinoylated proteins, which may contribute greatly to the development and progress of retinoylation research.

Many compounds induce differentiation of HL60 cells to granulocyte-like or monocyte/macrophage-like cells with inhibition of cell growth (*32*). For instance, RA and dimethyl sulfoxide (DMSO) induce HL60 cells to granulocyte-like cells. In contrast, 1α-25 dihydroxyvitamin D_3, butyric acid, interferon α and γ and mactinin induce HL60 cells to monocyte/macrophage-like cells. Mactinin is a urokinase degradation product of α-actinin that exhibits a molecular weight of 31 kDa (*33–35*). It has been reported that urokinase takes part in the differentiation of acute promyelocytic leukemias (HL60 cells, NB4 cells) to monocyte/macrophage-like cells (*34*). Urokinase is a well-known protease that catalyzes the conversion of plasminogen to plasmin, which is related to fibrin degradation. While it is not clear whether all degradation of proteins by urokinase affects induction of differentiation of HL60 cells directly, mactinin, formed from α-actinin by urokinase, is a factor inducing differentiation into monocyte/macrophage-like cells. On the other hand, previous studies have shown that RA (1 μM) increases α-actinin levels in HL60 cells after 5 days (*35*, *36*). RA-sensitive HL60 clones exhibit lower levels of α-actinin than parental HL60 cells, and α-actinin levels are increased by RA treatment (*36*). In contrast, RA-resistant HL60 clones exhibit higher levels of α-actinin than parental HL60 cells, and these levels are decreased by RA treatment (*36*). The current study shows that α-actinin was modified by RA, which is a factor inducing differentiation to granulocyte-like cells. α-Actinin may play a significant role in RA-induced differentiation. It would be interesting to examine intracellular location and extracellular releases of α-actinin and urokinase during RA-induced differentiation and whether amiloride, a specific inhibitor of urokinase, influences differentiation by RA in HL60 cells. The results of such studies may lead to an understanding of how retinoylated α-actinin may take part in the differentiation of HL60 cells to granulocyte-like cells and whether urokinase may be a key enzyme in determining the direction of differentiation. Further investigations are required to understand the physiological relevance of α-actinin and urokinase in RA-induced differentiation.

Recently, it has been reported that α-actinin may play a role in the transcriptional activation of nuclear receptors, such as steroids, glucocorticoids, thyroid and RA receptors (*37*). Screening has been conducted on mammalian proteins that bind to activation domains of activators and coactivators, which transmit transcriptional activation signals from DNA-bound nuclear receptors to chromatin and transcription machinery. One of these has been identified as α-actinin. α-Actinin contains an LXXLL motif near the amino acid terminus (amino acid 72–76) that binds to nuclear receptors. It is a primary coactivator of androgen, estrogen and thyroid receptors and it is an enhancer on these nuclear receptors with glucocorticoid receptor interacting protein 1. Thus, α-actinin interacts with nuclear receptors and their coactivators. However, the mechanisms of involvement of α-actinin in nuclear receptor activation are not understood. Recently, it was reported (*38*) that α-actinin can be located in the nucleus, depending on the cell type or state of actin depolymerization, and that an unidentified regulatory intracellular translocation of α-actinin may affect the final functional role of α-actinin in cells. The covalent binding of RA to α-actinin as shown in the current study may affect the activation potency of α-actinin on nuclear receptors or it may involve translocation of α-actinin into the nucleus. It would be interesting to examine whether retinoylated α-actinin is found in the nucleus and whether the binding affinity of unmodified α-actinin to activation domains of activator and coactivator are distinct from that of retinoylated α-actinin.

In this study, retinoylated proteins have been detected using ARMAs, and one of these proteins has been identified as α-actinin. Elucidating the components that interact with RA will be accelerated by this new methodology, thereby facilitating investigation of the mechanisms of RA actions. Further progress in the development of anticancer therapies should be possible based on clarifying the mechanisms of RA action.

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