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Formation of Retinoylated Proteins from Retinoyl-CoA in Rat Tissues

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Retinoylation (acylation of proteins by retinoic acid) is considered as one mechanism of retinoic acid (RA) action occurring in cells *in vitro* and *in vivo*. Previously, our studies showed that in rat tissues the formation of retinoyl-CoA from RA, the first step of retinoylation, required ATP, CoA and $MgCl_2$. In the current study, we examined whether the transfer of retinoyl-CoA into proteins, the second step of retinoylation, occurs in rat tissues. [^{3}H]-Labeled-retinoyl-CoA bound covalently to proteins in rat liver, kidney, testis, and brain. The levels of incorporation of retinoyl-CoA into proteins were higher in vitamin A–deficient rats than in normal ones. The formation of retinoylated proteins depended on the incubation time, and the concentrations of retinoyl-CoA and homogenate. The reaction was suppressed by fatty acyl-CoAs and palmitic acid, but not by arachidonic acid. The Vmax and Km values for retinoyl-CoA in the formation of retinoylated proteins using a crude liver extract were estimated to be 2,597.3 pmol/min/mg protein and 9.5×10^{-5} M, respectively. Retinoylated proteins formed from retinoyl-CoA, including a 17 kDa protein exhibiting high radioactivity, disappeared in the presence of 2-mercaptoethanol, indicating that RA was linked to the proteins through a thioester bond. These results demonstrate that retinoylation in rat tissues occurs *via* retinoyl-CoA formed from RA. This process may play a significant physiological role in cells.

Key words: retinoic acid, retinoyl-CoA, retinoylation, rat tissue, transfer.

Abbreviations: RA, retinoic acid; PBS, phosphate-buffered saline (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136.9 mM NaCl, pH 7.2); EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; DTT, dithiothreitol; ATP, adenosine triphosphate; CoA, coenzyme A; RAR, retinoic acid nuclear receptor.

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Retinoic acid (RA), a carboxylic acid derivative of vitamin A (retinol), plays many roles in mammalian cells and tissues. Induction of differentiation and inhibition of growth in human leukemia cells are well known as representative biological effects of RA (*1*). Clarification of the mechanisms of the RA action is of critical interest, since various biological effects of RA have been reported.

As one mechanism for the action of RA, binding to RA nuclear receptors [retinoic acid receptors (RARs) or retinoid X receptors (RXRs)] has been reported (*2–6*). These receptors mediate the action of RA, which directly regulates the transcription of target genes by binding to specific DNA sequences. However, not all the effects of RA can be explained by nuclear receptor pathways (*7–12*). Other mechanisms, in addition to RA receptors, may be involved in RA biological effects.

Retinoylation (acylation of proteins by RA) is another mechanism of the RA action (*13–17*). RA induces terminal differentiation of the human acute myeloid leukemia cell line HL60 into cells similar to mature granulocytes (*18*). In growing HL60 cultures, RA is linked *via* a thioester bond to proteins, and the extent of retinoylation depends in a saturable manner on the initial concentration of RA (*14*, *17*). Dose-response curves for RA-induced differentiation and retinoylation are similar and exhibit positive correlations (*14*, *17*). The regulatory subunits of cyclic AMP–dependent protein kinase types I and II and vimentin in HL60 cells, and the cytokeratins in normal human keratinocytes are retinoylated (*15*, *16*, *19*). In addition, we have shown that retinoylation occurs *in vivo* primarily *via* the formation of an ester bond (*20*). Thus, retinoylation may be a true physiological reaction of RA.

Retinoylation is one of several post-translational modifications affecting many proteins, including phosphorylation, palmitoylation, and myristoylation. Palmitoylation and myristoylation are known to involve the formation of CoA derivatives and the transfer of acyl-CoA to proteins, resulting in the acylation of proteins (*21*, *22*). Recently, the enzymatic formation of retinoyl-CoA from RA has been demonstrated in crude extracts of rat tissues (*23*, *24*). However, the second step of retinoylation after retinoyl-CoA formation has not been shown.

Previously, the formation of retinoylated proteins from retinoyl-CoA in subcellular fractions (membrane/nuclei, mitochondria, microsome, and cytosol) of vitamin A–deficient rat kidney was studied (*24*). The latter study demonstrated that the incorporation of retinoyl-CoA into proteins was enzymatic judging from the heat inactivation and SDS denaturation of subcellular fractions containing enzyme or/and protein substrates. In the present study, we investigate the transfer of retinoyl-CoA to proteins in rat tissue homogenates *in vitro* using a cell free system.

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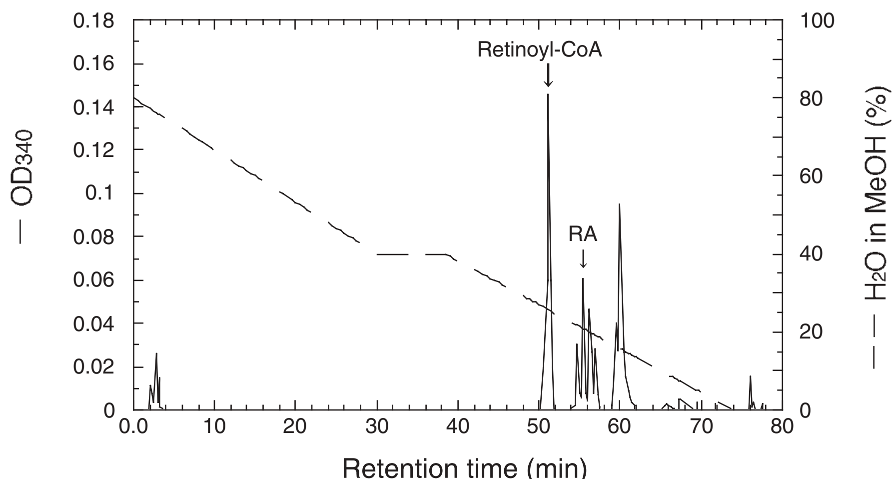

Fig. 1. **HPLC profile of retinoyl-CoA.** A Spherisorb C8 column (4.6 mm × 2.5 cm; Phenomenex) was eluted with 10 mM NH_4OAc in a methanol/water gradient system at a flow rate of 1.0 ml/min with UV monitoring at 340 nm. The arrows indicate the elution positions of retinoyl-CoA (retention time, 51.1 min) and all-*trans*-retinoic acid (retention time, 57.0 min).

MATERIALS AND METHODS

Animals—Eight-week-old male Sprague-Dawley (Slc. SD) rats (140–160 g) were obtained from Tokyo Laboratory Animals Science (Tokyo). Three-week-old male SD rats (50 g) were maintained on a vitamin A–deficient diet (CLEA, Tokyo) for 14 weeks (250 g) as described previously (*20*).

Preparation of Homogenates of Rat Tissues—The following procedures were performed at 0–4°C as described previously (*23–25*). Rat liver, kidney, testis, and brain tissues were each homogenized in 5 volumes (w/v) of Buffer A [100 mM Tris-HCl buffer (pH 7.4), 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), and proteinase inhibitors [0.5 mM phenylmethylsulfonyl fluoride, 0.4 mg of aprotinin/ml, 0.2 mg of leupeptin/ml, and 15 μM 4-amidinophenylmethane-sulfonyl fluoride (Sigma)].

Protein Assay—Proteins were measured using bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL) or the procedure of Bradford (*26*) with bovine serum albumin as a standard.

Preparation of Retinoyl-CoA—Labeled or radioinert retinoyl-CoA was synthesized and identified according to the modification of the method of Kutner *et al*. (*27*). Briefly, all-*trans*-[^{3}H]RA ([11,12-^{3}H]-RA, 35.8–50 Ci/mmol (1.11 TBq/mmol); 1 Ci = 3.7×10^{10} Bq; Du Pont–New England Nuclear, Boston, MA) or radioinert RA (2 μmol) was dissolved in dioxane, diluted to a final concentration of 2.5 mM with an equimolar amount (2 μmol) of butylated hydroxytoluene (BHT) dissolved in dioxane, and then taken to dryness three times from dioxane under argon gas. This preparation was treated with 400 μl (8 μmol) of 20 mM *N*-hydroxysuccinimide and 400 μl (8 μmol) of 20 mM *N,N′*-dicyclohexylcarbodiimide dissolved in dioxane, and then the reaction mixture was incubated at 37°C for 1 h under argon gas. After removing dioxane under an argon stream, 1% $NaHCO_3$ (0.5 ml) was added to the dry ester dissolved in tetrahydrofuran (THF) (1 ml), the resulting solution was added to a CoA solution [15 μmol (12.3 mg), 0.1 ml], and then the pH was adjusted slowly to 8.0–8.5 by the addition of 1% $NaHCO_3$ (0.4 ml). The ratio of THF and water was two to one. The solution was mixed well and then incubated at 37°C with stirring under argon overnight. The reaction was stopped by adding 1 ml of $CHCl_3$/MeOH (2:1) containing BHT (50 μg/ml) and mixing well, and then the

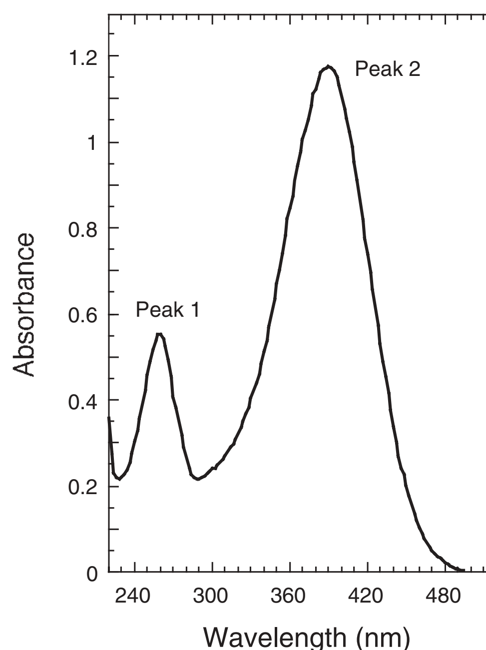

Fig. 2. **UV absorption spectrum of all-*trans*-retinoyl CoA.** λ, wave length; ε, extinction coefficient. Peak 1: λ_1 = 258 nm, ε = 1.1×10^4, Peak 2: λ_2 = 393 nm, ε = 3.4×10^4.

mixture was centrifuged at 1,000 × *g* for 10 min. The upper aqueous layer was lyophilized to remove the solvent and the residue was dissolved in 200 μl of distilled water. The purity of the aqueous product was estimated and the product identified by high pressure liquid chromatography (HPLC) (Fig. 1). A peak corresponding to retinoyl-CoA was collected and a UV spectrum was measured with a DU 530 Life Science UV/Vis Spectrophotometer (Beckman Instruments Arlington Heights, IL) (Fig. 2).

Formation of Retinoylated Proteins from Retinoyl-CoA—Homogenates (0–1,000 μg protein) prepared from rat liver, kidney, brain and testis were incubated for 5 min at 37°C with 8 nmol [^{3}H]retinoyl-CoA (6 mCi/mmol), and reaction buffer [0.1 M Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol and, 50 mM sucrose] in a final volume of 0.1 ml. The reactions were terminated by the addition

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of 1.04 ml of $CHCl_3$/MeOH/H_2O (1:2:0.46), and then centrifugation at 10,000 × *g* for 5 min. After centrifugation, the pellets were extracted by the modified Bligh-Dyer procedure (*28*), followed by centrifugation at 10,000 × *g* for 5 min. This extraction was repeated five times or until there was <100 cpm/0.2 ml in the supernatant. The delipidated pellets were then dried in a centrifugal vacuum device and dissolved in SOLVABLE (Packard Instrument). Radioactivity was measured on a liquid scintillation spectrometer (Packard Instrument). Homogenates boiled at 100°C for 10 min or having $CHCl_3$/MeOH/H_2O (1:2:0.46) added before the reaction were used as negative controls. The amounts of retinoylated proteins formed from retinoyl-CoA were calculated by subtracting the radioactivity of a control from the radioactivity of a sample.

Separation of Retinoylated Proteins Formed from Retinoyl-CoA by SDS–Polyacrylamide Gel Electrophoresis—Liver homogenates (10 μg) were incubated for 5 min at 37°C with 8 nmol of [^{3}H]retinoyl-CoA (6 mCi/mmol), and reaction buffer [0.1 M Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50 mM sucrose] in a final volume of 0.1 ml. The reactions, were terminated by the addition of 1.04 ml of $CHCl_3$/MeOH/H_2O (1:2:0.46), and then centrifugation at 10,000 × *g* for 5 min. The pellets were then extracted by the Bligh-Dyer modified procedure and centrifuged at 10,000 × *g* for 5 min. This extraction was repeated about five times or until there was <100 cpm/0.2 ml in the supernatant. The delipidated proteins were dissolved in sample buffer [62.5 mM Tris-HCl (pH 6.8) containing 2% (w/v) SDS, 10% (v/v) glycerol and 0.01% bromphenol blue with or without 2-mercaptoethanol], and then separated by 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) using the discontinuous buffer system of Laemmli (*29*). Proteins in the gel were electrically transferred to a polyvinylidene difluoride membrane by means of a semi dry transfer device (Bio craft) using transfer buffer [40 mM Tris-glycine, 20% methanol and 0.1% (w/v) SDS]. Membranes after transfer were sliced (0.2 × 1 cm), and then radioactivity was measured on a liquid scintillation spectrometer (Packard Instrument).

RESULTS

Dose Response and Time Course of the Formation of Retinoylated Proteins from Retinoyl-CoA—We initially examined whether the transfer of retinoyl-CoA to proteins occurred in various rat tissues. The levels of retinoylated proteins, produced on the incubation of [^{3}H]retinoyl-CoA and homogenates of liver, kidney, testis and brain for 5 min, were measured. The amounts of retinoylated proteins formed from retinoyl-CoA were calculated by subtracting the control radioactivity from the sample radioactivity. The level of proteins incorporating retinoyl-CoA decreased markedly when tissues were boiled or treated with an organic solvent, suggesting that the retinoyl-CoA incorporation into proteins was enzymatic.

As shown in Fig. 3, the transfer of retinoyl-CoA into proteins was observed with various homogenate concentrations (10–1,000 μg) in liver, kidney, testis and brain. The amounts of retinoylated proteins formed from retinoyl-CoA increased linearly with the amount of protein up to 500 μg for liver, 250 μg for kidney and 500 μg for brain, at which point saturation began. On the other hand, in testis, the formation of retinoylated proteins increased up to 250 μg of crude extract, and remained constant thereafter.

Next, the time dependency of the production of retinoylated proteins from retinoyl-CoA was examined using homogenates of tissues and [^{3}H]retinoyl-CoA. We measured the formation of retinoylated proteins from retinoyl-CoA in crude extracts of liver, kidney, testis, and brain at various time periods (Fig. 4). The amounts of retinoylated proteins increased in a time-dependent manners. Linear increases in retinoylated protein production were observed within incubation times of 10 min, 2 min, 5 min, and 2 min in liver, kidney, brain, and testis crude extracts, respectively. In particular, retinoylated proteins in kidney and testis homogenates formed quickly and linearly for only a short period. In addition, the level of retinoylated proteins in testis remained constant after the initial 2 min incubation.

Effect of the Retinoyl-CoA Concentration on Formation of Retinoylated Proteins from Retinoyl-CoA—As shown in Fig. 5, the formation of retinoylated proteins from retinoyl-CoA depended in a linear manner on the concentration of retinoyl-CoA within the concentration range of 0 μM to 80 μM, the V_{max} and K_m values being 2,597.3 pmol/min/mg and 9.5×10^{-5} M, respectively.

Separation of Retinoylated Proteins from Retinoyl-CoA by SDS-PAGE—While the formation of retinoylated proteins from retinoyl-CoA was confirmed in crude extracts of liver, kidney, brain and testis, the identity of the retinoylated proteins was unclear. Therefore, [^{3}H]retinoylated proteins in rat liver were prepared by means of an *in vitro* reaction using [^{3}H]retinoyl-CoA, and separated by SDS-PAGE in the presence or absence of 2-mercaptoethanol. In the absence of 2-mercaptoethanol, radioactive retinoylated proteins were observed in the range of 15 kDa to 111 kDa (Fig. 6A). Among these proteins, the highest incorporation of retinoyl-CoA was seen in proteins exhibiting molecular weights of approximately 17 kDa. In contrast, radioactive retinoylated proteins including 17 kDa ones decreased markedly in the presence of 2-mercaptoethanol (Fig. 6B). These results indicated that retinoylated proteins derived from retinoyl-CoA bound to proteins through thioester bonds.

Effects of Fatty Acyl-CoAs and Fatty Acids on the Formation of Retinoylated Proteins—RA consists of a cyclohexene ring connected to a conjugated long-chain tetra-ene carboxic acid, which is converted to retinoyl-CoA. On the assumption that fatty acyl-CoAs and fatty acids inhibit retinoyl-CoA incorporation into proteins, the effects of acyl-CoAs (acetyl-CoA, acetoacetyl-CoA, myristoyl-CoA, palmitoyl-CoA and stearoyl-CoA) and fatty acids (palmitic acid and arachidonic acid) were examined using rat liver homogenates. As shown in Table 1, the generation of retinoylated proteins in rat liver was inhibited approximately 60% by acetyl-CoA, acetoacetyl-CoA, myristoyl-CoA, and palmitoyl-CoA, and approximately 20% by stearoyl-CoA at a concentration of 100 μM. At a higher concentration (240 μM), the inhibitory effects of all acyl-CoAs increased. These results indicated that short-chain and long-chain acyl-CoAs affect the production of retinoylated proteins from retinoyl-CoA.

On the other hand, palmitic acid (C16:0) inhibited retinoyl-CoA incorporation into proteins by approximately

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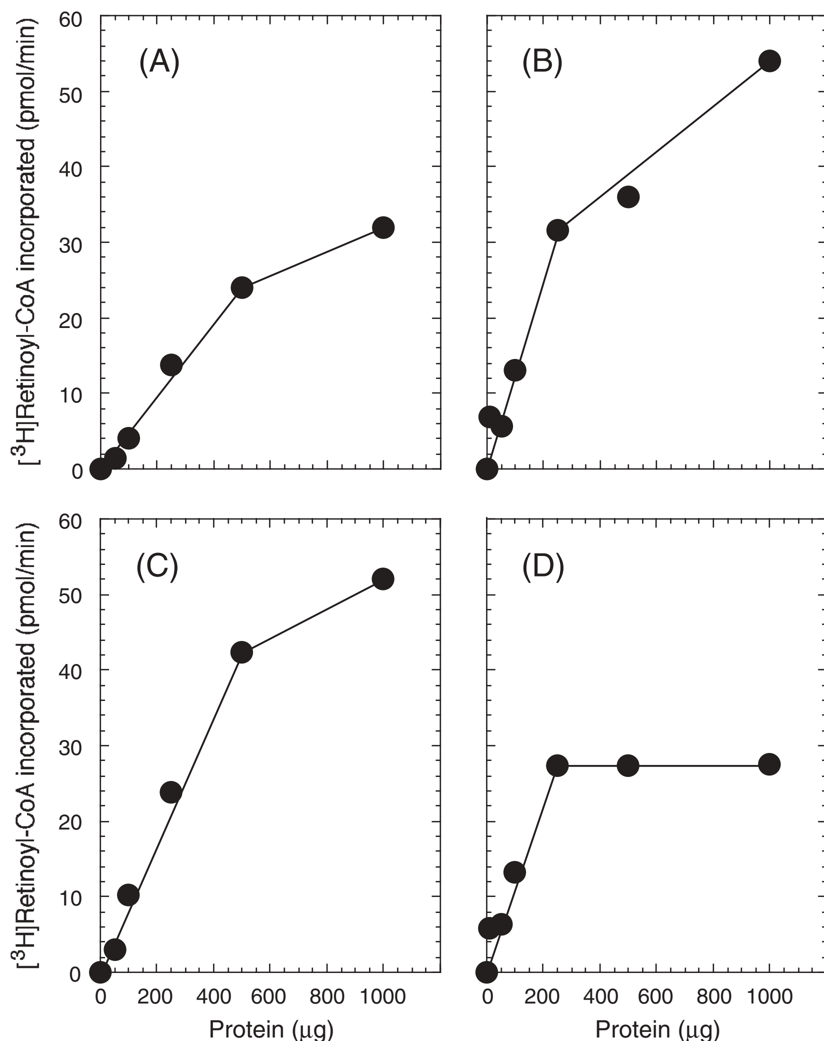

Fig. 3. **Dependence of retinoyl-CoA transfer activity on protein concentration.** Homogenates prepared from rat liver (A), kidney (B), brain (C), and testis (D) were incubated for 5 min at 37°C with 8 nmol [^{3}H]retinoyl-CoA (6 mCi/mmol), 0.1 M Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50 mM sucrose in a final volume of 0.1 ml. Each reaction mixture contained the indicated concentration of protein. Reactions were terminated by the addition of 1.04 ml of $CHCl_3$/MeOH/H_2O (1:2:0.46) and then centrifugation at $10{,}000 \times g$ for 5 min. After centrifugation, each pellet was extracted by the Bligh-Dyer modified procedure and centrifuged at $10{,}000 \times g$ for 5 min. This extraction was repeated approximately five times or until there was <100 cpm/0.2 ml in the supernatant. The delipidated pellets were then dried in a centrifugal vacuum device and dissolved in SOLVABLE (Packard). Radioactivity was measured using a liquid scintillation spectrometer.

20% at 100 μM and approximately 75% at 240 μM, as compared with approximately 35% and approximately 90% inhibition caused by palmitoyl-CoA. However, arachidonic acid (C20:4) enhanced the retinoylated protein level approximately 2-fold at a concentration of 240 μM. These results suggested that the specificity and potency of inhibition may be due to the three-dimensional structures of the fatty acids, with dependence on both the carbon chain length and double bond position.

Formation of Retinoylated Proteins from Retinoyl-CoA in Vitamin A–Deficient Rat Liver—Previous studies showed that the action of RA is more pronounced in tissues of vitamin A–deficient rats than in normal ones, and that the amounts of retinoylated proteins generated from RA in vitamin A–deficient rats are higher than in normal ones. These findings let us to examine the production of retinoylated proteins from retinoyl-CoA in tissues of normal and vitamin A–deficient rats. As shown in Table 2, the incorporation of retinoyl-CoA into proteins in normal rat liver was approximately one half of that in vitamin A–deficient rat liver. These results indicated that a strong positive correlation exists between retinoylation from retinoyl-CoA and the RA action.

DISCUSSION

RA is used as a potent inducer of differentiation of both cell lines and cells from leukemia patients (*1*, *18*, *30*). Previous studies have shown that in HL60 cells RA nuclear receptors or retinoylation may mediate RA-induced differentiation and that retinoylation may occur *via* enzymatic retinoyl-CoA formation from RA (*23*).

In the current study, we synthesized radiolabelled or radioinert retinoyl-CoA, and examined whether retinoyl-CoA was transferred to proteins during the retinoylation process. The formation of retinoylated proteins from retinoyl-CoA occurred in rat tissues, and was dependent on the crude extract and retinoyl-CoA concentrations, and the incubation time. In addition, the K_m value for transfer of retinoyl-CoA to proteins with a crude rat liver extract was 9.5×10^{-5} M and the V_{max} value was 2,597.3 pmol/min/mg. While various proteins including 17 kDa ones, which exhibited the highest radioactivity, were modified by retinoyl-CoA, the amounts of these retinoylated proteins decreased markedly in the presence of 2-mercaptoethanol.

It had previously been shown that acylation, or the transfer of fatty acids into proteins affects several cellular signal transductions processes (*31*). One type of fatty acylation,

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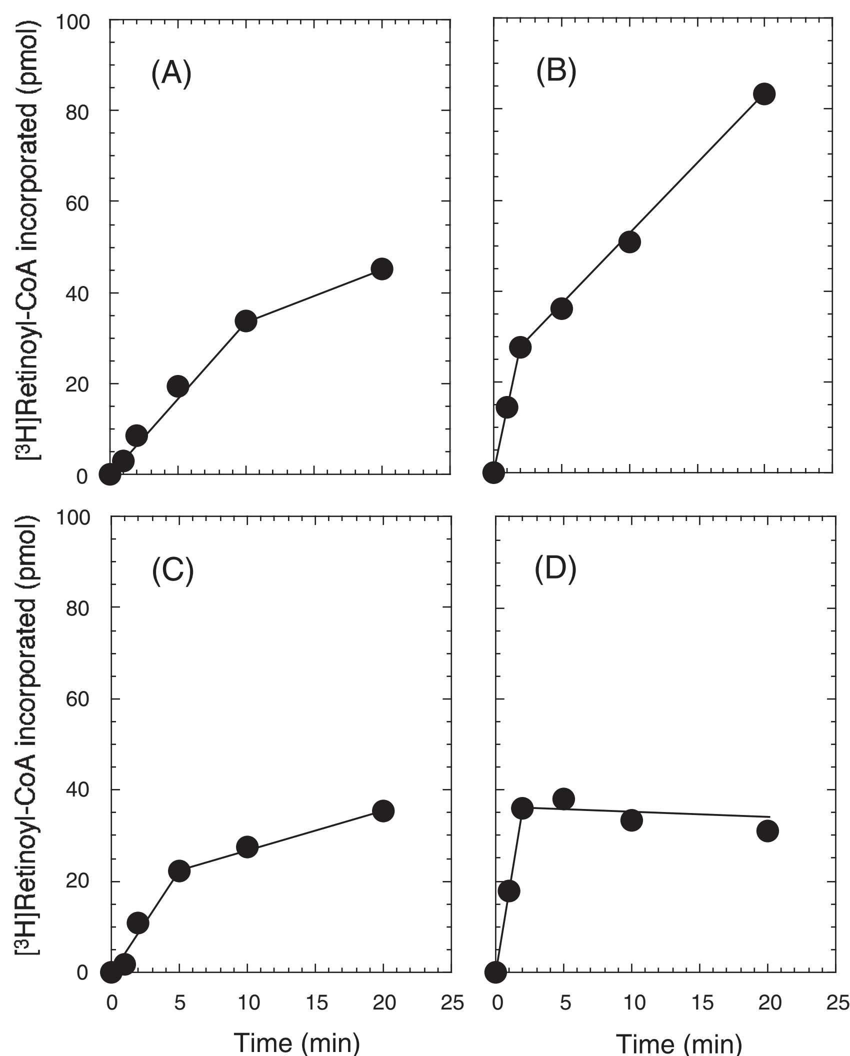

Fig. 4. **Time course of the retinoyl-CoA transfer reaction.** Homogenates (50 μg) prepared from rat liver (A), kidney (B), brain (C), and testis (D) were incubated for the indicated times at 37°C with 8 nmol [^{3}H]retinoyl-CoA (6 mCi/mmol), 0.1 M Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50 mM sucrose in a final volume of 0.1 ml. Reactions were terminated by the addition of 1.04 ml of $CHCl_3/MeOH/H_2O$ (1:2:0.46) and then centrifugation at 10,000 × *g* for 5 min. After centrifugation, each pellet was extracted by the Bligh-Dyer modified procedure and centrifuged at 10,000 × *g* for 5 min. This extraction was repeated approximately five times or until there was <100 cpm/0.2 ml in the supernatant. The delipidated pellets were then dried in a centrifugal vacuum device and dissolved in SOLVABLE (Packard). Radioactivity was measured using a liquid scintillation spectrometer. Samples were incubated for the indicated times at 37°C.

myristoylation, occurs through enzymatic transfer of myristic acid into proteins *via* myristoyl-CoA as an intermediate (*31*, *32*). In contrast, the enzymology of palmitoylation is poorly understood. In the current study, the incorporation of retinoyl-CoA into proteins was prevented by boiling the tissues and by treatment with organic solvents. In addition, the generation of retinoylated proteins with retinoyl-CoA increased in a fashion dependent on the concentration of retinoyl-CoA, the incubation time and the homogenate concentration in a saturable manner. These results suggested that the transfer of retinoyl-CoA into proteins may be enzymatic.

Cione and Genchi (*33*) have reported that the K_m value for retinoylation by RA (RA → retinoylated protein) with rat testis mitochondria is 700 ± 75 nM, and that the V_{max} is 100 ± 18 pmol/mg protein/90 min (1.11 ± 0.2 pmol/min/mg protein). In contrast, Wada *et al*. (*23*) have shown that the K_m value for the formation of retinoyl-CoA from RA (RA → retinoyl-CoA) with rat liver microsomes is 24 nM, the V_{max} value being 100 pmol/min/mg protein. In the current study, retinoylation with retinoyl-CoA of proteins (retinoyl-CoA → retinoylated proteins) using a crude rat liver extract yielded a K_m value of 9.5×10^{-5} M and a V_{max} value of 2,597.3 pmol/min/mg protein. These K_m and V_{max} values for retinoylation by RA were between those for retinoyl-CoA formation and retinoyl-CoA transfer, although the conditions, including the tissue extracts and fractions, used for the three reactions were different. It is possible that on retinoylation, retinoyl-CoA may be generated from RA extremely quickly, and that the covalent-bond formation transfer of retinoyl-CoA into proteins may be rate limiting. Further studies under the same conditions are required to examine this possibility.

In Figs. 3 and 4, the formation of retinoylated proteins with retinoyl-CoA was dependent on time and the homogenate concentration for rat liver, kidney, brain, and testis. The reaction rates, from high to low, were in the order of kidney, testis, brain, and liver. The reaction patterns for kidney and testis were distinct from each other, while the production of retinoylated proteins was rapid (Figs. 3D and 4D). Incorporation of retinoyl-CoA into proteins ceased sooner in testis as compared to in kidney. This rapid decrease in testis may be because depletion of retinoyl-CoA occurs as a result of the catabolism by CoA thiolase. The depletion of specific cellular proteins or the presence of inhibitors is unlikely. Further experiments regarding the stability of retinoyl-CoA in different extracts are necessary to clarify this issue.

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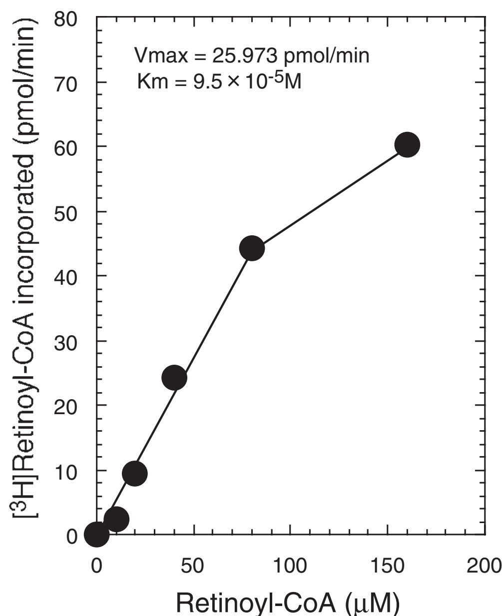

Fig. 5. **Dependence of retinoyl-CoA transfer activity on [^{3}H]retinoyl-CoA concentration.** Liver homogenates (10 μg) were incubated for 5 min at 37°C with the indicated concentrations of 8 nmol [^{3}H]retinoyl-CoA (6 mCi/mmol), 0.1 M Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50 mM sucrose in a final volume of 0.1 ml. Reactions were terminated by the addition of 1.04 ml of $CHCl_3/MeOH/H_2O$ (1:2:0.46) and then centrifugation at 10,000×g for 5 min. After centrifugation, each pellet was extracted by the Bligh-Dyer modified procedure and centrifuged at 10,000×g for 5 min. This extraction was repeated approximately five times or until there was <100 cpm/0.2 ml in the supernatant. The delipidated pellets were then dried in a centrifugal vacuum device and dissolved in SOLVABLE (Packard). Radioactivity was measured using a liquid scintillation spectrometer.

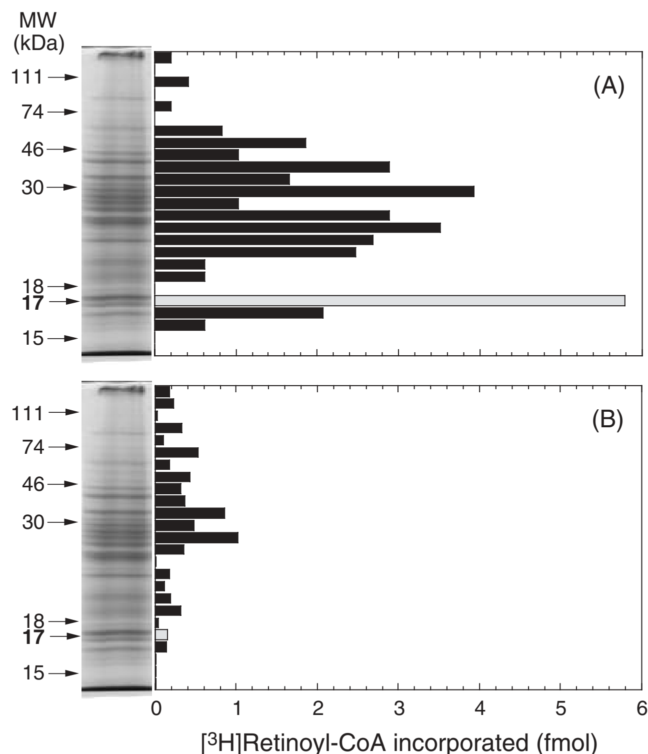

Fig. 6. **SDS-PAGE of retinoylated proteins obtained after vitro reactions.** Liver homogenates (10 μg) were incubated for 5 min at 37°C with 8 nmol of [^{3}H]retinoyl-CoA (6 mCi/mmol), 0.1 M Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50 mM sucrose in a final volume of 0.1 ml. Reactions were terminated by the addition of 1.04 ml of $CHCl_3/MeOH/H_2O$ (1:2:0.46) and then centrifugation at 10,000×g for 5 min. Each pellet was then extracted by the Bligh-Dyer modified procedure and centrifuged at 10,000 × g for 5 min. This extraction was repeated approximately five times or until there was <100 cpm/0.2 ml in the supernatant. The delipidated pellets were dissolved in 62.5 mM Tris-HCl containing 2% (w/v) SDS, 10% (v/v) glycerol, and 0.01% bromphenol blue, in the absence (A) or presence (B) of 2-mercaptoethanol, and then loaded onto a 10% SDS–polyacrylamide gel. SDS-PAGE was performed using the discontinuous buffer system of Laemmli. Proteins in the gels were electrically transferred to polyvinylidene difruoride membranes by means of a semidry transfer device (Biocraft) using a transfer buffer [40 mM Tris-glycine, 20% methanol and 0.1% (w/v) SDS]. After transfer, the membranes were sliced, 0.2 × 1 cm, and then radioactivity was measured using a liquid scintillation spectrometer. On the left side are Coomassie Blue-stained gels.

Retinoylated proteins prepared from [^{3}H]retinoyl-CoA with rat liver extracts *in vitro* exhibited molecular weights in the range of 15 kDa to 111 kDa (Fig. 4A). The highest incorporation of retinoyl-CoA occurred in proteins with molecular weights around 17 kDa. Treatment with 2-mercaptoethanol of most retinoylated proteins including 17 kDa ones released radioactivity (Fig. 4B). These data indicated that most of the linkages between the retinoyl residues and proteins may be thioester bonds. This finding coincides with the report by Myhre *et al*. (*20*) that the size of retinoylated proteins in vitamin A–deficient rat liver *in vivo* is approximately 16–17 kDa and that these retinoylated proteins are formed *via O*-ester or thioester bonds. These studies showed that a 17 kDa protein was the major retinoylated protein in liver, and that the amount of this protein was extremely small. While purification of the 17 kDa–retinoylated protein may be difficult, it would be interesting to see which protein was retinoylated. Identification of the 17 kDa–retinoylated protein in rat liver is underway.

Table 2 shows that the formation of retinoylated proteins with a liver homogenate in vitamin A–deficient rats was approximately two-fold higher than that in normal rats. This is in agreement with previous studies showing that the actions of RA and the amounts of retinoylated proteins generated from RA in vitamin A–deficient rats are higher than in normal rats (*14*, *17*, *20*). This finding suggests that retinoylation by retinoyl-CoA exhibits a strong positive correlation with the RA action.

A previous study has shown that the synthesis of retinoyl-CoA was suppressed by fatty acids and fatty acyl-CoAs (*23*). Arachidonic acid and palmitic acid inhibited the production of retinoyl-CoA markedly. In contrast, myristoyl-CoA, palmitoyl-CoA and stearoyl-CoA (long-chain fatty acyl-CoAs) suppressed the production of retinoyl-CoA in a manner proportional to increasing acyl-CoA concentration. The percent inhibition by both acetyl-CoA and acetoacetyl-CoA (short-chain fatty acyl-CoAs) was less than that by long-chain fatty acyl-CoAs. These results indicated that hydrophobic residues consisting of a cyclohexene ring connected to a conjugated

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Table 1. **Inhibition of retinoyl-CoA incorporation into proteins by fatty acyl-CoAs and fatty acids.** The levels of retinoyl-CoA incorporation into proteins were measured as described under "MATERIALS AND METHODS." Rat liver homogenates (10 μg) were incubated for 5 min at 37°C with 8 nmol of [^{3}H]retinoyl-CoA (6 mCi/mmol), 0.1 M Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50 mM sucrose in a final volume of 0.1 ml in the absence or presence of fatty acyl-CoAs (acetyl-CoA, acetoacetyl-CoA, myristoyl-CoA, palmitoyl-CoA, and stearoyl-CoA) and fatty acids (palmitic acid and arachidonic acid).

	Concentration (μM)	
	100	240
None	100	100
Acetyl-CoA	42.4	27.3
Acetoacetyl-CoA	45.0	2.1
Myristoyl-CoA	43.8	9.5
Palmitoyl-CoA	34.6	9.3
Stearoyl-CoA	80.3	57.5
Palmitic acid	79.2	25.8
Arachidonic acid	132.6	200.8

Table 2. **Retinoyl-CoA incorporation to proteins in normal and vitamin A–deficient rat liver.** The levels of retinoyl-CoA incorporation into proteins were measured as described under "MATERIALS AND METHODS." Liver homogenates (250 μg) from normal and vitamin A–deficient rats were incubated for 5 min at 37°C with 8 nmol of [^{3}H]retinoyl-CoA (6 mCi/mmol), 0.1 M Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50 mM sucrose in a final volume of 0.1 ml. Values are expressed as means ± SE.

	[^{3}H]retinoyl-CoA incorporated (pmol/mg/min)
Normal	56 ± 5
Vitamin A–deficiency	104 ± 8

long-chain tetra-ene carboxic acid affect retinoyl-CoA formation from RA. In the current study, the production of retinoylated proteins with retinoyl-CoA was affected by both short-chain and long-chain acyl-CoAs, suggesting that the CoA residue was significant for the inhibition of retinoyl-CoA incorporation into proteins (Table 1). On the other hand, palmitic acid inhibited the formation of retinoylated proteins with retinoyl-CoA, although less than by palmitoyl-CoA (Table 1). However, arachidonic acid enhanced the retinoylated protein levels approximately two-fold. Further studies are required to determine which interactions between fatty acids and retinoyl-CoA or substrate proteins are critical for the specificity and potency of inhibition on the generation of retinoylated proteins.

Retinoylation is a posttranslational modification of proteins that occurs in a variety of cell types *in vitro* and in tissues *in vivo*. This suggests that it may play a role in many effects of RA on cells. One metabolic pathway for retinoylation is the intermediate formation of retinoyl-CoA, and subsequent transfer and covalent binding of the retinoyl moiety to a protein. In previous studies on retinoylation, subcellular fractions were used and the levels of retinoylated proteins formed from RA or retinoyl-CoA were measured (*24*, *33*, *34*). Such studies are lacking because two enzymes and substrate proteins are necessary in order for retinoylation to occur. Retinoylation is apparently a true physiological reaction of RA in cells. Identification of retinoylated proteins may shed light on the role of retinoylation.

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REFERENCES

1. Huang, M.E., Ye, Y.C., Chen, S.R., Chai, J.R., Lu, J.X., Zhoa, L., Gu, L.J., and Wang, Z.Y. (1988) Use of all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia. *Blood* **72**, 567–572
2. Kastner, P., Mark, M., and Chambon, P. (1995) Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell* **83**, 859–869
3. Chambon, P. (1996) A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10**, 940–954
4. Mangelsdorf, D.J. and Evans, R.M. (1995) The RXR heterodimers and orphan receptors. *Cell* **83**, 841–850
5. Wolf, G. (2000) Cellular retinoic acid-binding protein II: a coactivator of the transactivation by the retinoic acid receptor complex RAR.RXR. *Nutr. Rev.* **58**, 151–153
6. Rowe, A. (1997) Retinoid X receptors. *Int. J. Biochem. Cell Biol.* **29**, 275–278
7. Bolmer, S.D. and Wolf, G. (1982) Retinoids and phorbol esters alter release of fibronectin from enucleated cells. *Proc. Natl. Acad. Sci. USA* **79**, 6541–6545
8. Smith, T.J., Davis, F.B., and Davis, P.J. (1992) Stereochemical requirements for the modulation by retinoic acid of thyroid hormone activation of Ca^{2+}-ATPase and binding at the human erythrocyte membrane. *Biochem. J.* **284**, 583–587
9. Smith, T.J., Davis, F.B., Deziel, M.R., Davis, P.J., Ramsden, D.B., and Schoenl, M. (1994) Retinoic acid inhibition of thyroxine binding to human transthyretin. *Biochim. Biophys. Acta* **1199**, 76–80
10. Crowe, D.L. (1993) Retinoic acid mediates post-transcriptional regulation of keratin 19 mRNA levels. *J. Cell Sci.* **106**, 183–188
11. Djakoure, C., Guibourdenche, J., Porquet, D., Pagesy, P., Peillon, F., Li, J.Y., and Evain-Brion, D. (1996) Vitamin A and retinoic acid stimulate within minutes cAMP release and growth hormone secretion in human pituitary cells. *J. Clin. Endocrinol. Metab.* **81**, 3123–3126
12. Varani, J., Burmeister, B., Perone, P., Bleavins, M., and Johnson, K.J. (1995) All-*trans* retinoic acid inhibits fluctuations in intracellular Ca^{2+} resulting from changes in extracellular Ca^{2+}. *Am. J. Pathol.* **147**, 718–727
13. Takahashi, N. and Breitman, T.R. (1989) Covalent binding of 17 beta-estradiol and retinoic acid to proteins in the human breast cancer cell line MCF-7. *In Vitro Cell Dev. Biol.* **25**, 1199–1200
14. Takahashi, N. and Breitman, T.R. (1989) Retinoic acid acylation (retinoylation) of a nuclear protein in the human acute myeloid leukemia cell line HL60. *J. Biol. Chem.* **264**, 5159–5163
15. Takahashi, N., Liapi, C., Anderson, W.B., and Breitman, T.R. (1991) Retinoylation of the cAMP-binding regulatory subunits of type I and type II cAMP-dependent protein kinases in HL60 cells. *Arch. Biochem. Biophys.* **290**, 293–302
16. Takahashi, N., Jetten, A.M., and Breitman, T.R. (1991) Retinoylation of cytokeratins in normal human epidermal keratinocytes. *Biochem. Biophys. Res. Commun.* **180**, 393–400
17. Takahashi, N. and Breitman, T.R. (1991) Retinoylation of proteins in leukemia, embryonal carcinoma, and normal kidney cell lines: differences associated with differential responses to retinoic acid. *Arch. Biochem. Biophys.* **285**, 105–110

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18. Breitman, T.R., Selonick, S.E., and Collins, S.J. (1980) Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc. Natl. Acad. Sci. USA* **77**, 2936–2940
19. Takahashi, N. and Breitman, T.R. (1994) Retinoylation of vimentin in the human myeloid leukemia cell line HL60. *J. Biol. Chem.* **269**, 5913–5917
20. Myhre, A.M., Takahashi, N., Blomhoff, R., Breitman, T.R., and Norum, K.R. (1996) Retinoylation of proteins in rat liver, kidney, and lung *in vivo*. *J. Lipid Res.* **37**, 1971–1977
21. Schultz, A.M., Henderson, L.E., and Oroszlan, S. (1988) Fatty acylation of proteins. *Annu. Rev. Cell Biol.* **4**, 611–647
22. Towler, D.A., Gordon, J.I., Adams, S.P., and Glaser, L. (1988) The biology and enzymology of eukaryotic protein acylation. *Annu. Rev. Biochem.* **57**, 69–99
23. Wada, M., Fukui, T., Kubo, Y., and Takahashi, N. (2001) Formation of retinoyl-CoA in rat tissues. *J. Biochem.* **130**, 457–463
24. Renstrom, B. and DeLuca, H.F. (1989) Incorporation of retinoic acid into proteins via retinoyl-CoA. *Biochim. Biophys. Acta* **998**, 69–74
25. Ito, M., Fukui, T., Kamokari, M., Saito, T., and Tomita, K. (1984) Purification and characterization of acetoacetyl-CoA synthetase from rat liver. *Biochim. Biophys. Acta* **794**, 183–193
26. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
27. Kutner, A., Renstrom, B., Schnoes, H.K., and DeLuca, H.F. (1986) Synthesis of coenzyme A ester of retinoic acid: intermediate in vitamin A metabolism. *Proc. Natl. Acad. Sci. USA* **83**, 6781–6784
28. Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917
29. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
30. Breitman, T.R., Collins, S.J. and Keene, B.R. (1981) Terminal differentiation of human promyelocytic leukemic cells in primary culture in response to retinoic acid. *Blood* **57**, 1000–1004
31. Resh, M.D. (1999) Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim. Biophys. Acta* **1451**, 1–16
32. Smotrys, J.E. and Linder, M.E. (2004) Palmitoylation of intracellular signaling proteins: regulation and function. *Annu. Rev. Biochem.* **73**, 559–587
33. Cione, E. and Genchi, G. (2004) Characterization of rat testes mitochondrial retinoylating system and its partial purification. *J. Bioenerg. Biomembr.* **36**, 211–217
34. Genchi, G. and Olson, J.A. (2001) Retinoylation of proteins in cell-free fractions of rat tissues in vitro. *Biochim. Biophys. Acta* **1530**, 146–154

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