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Changes in Carboxylesterase Isoenzymes of Rat Liver Microsomes during Hepatocarcinogenesis

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Among the three major carboxylesterase isoenzymes, RH1, RL1 and RL2, present in microsomes from normal rat liver, RL2 shows hydrolyzing activity towards 12-*O*-tetradecanoylphorbol-13-acetate and 1-oleoyl-2-acetyl-*rac*-glycerol, both activators of protein kinase C. Since protein kinase C has been suggested to be involved in carcinogenesis and cell proliferation, alterations in hepatic microsomal carboxylesterase isoenzymes including RL2 were studied during hepatocarcinogenesis induced by the Solt-Farber model. Alteration of RL2 was determined by measuring acetanilide-hydrolyzing activity, by quantifying the protein amount using the single radial immunodiffusion method, and by activity staining following electrophoresis of liver microsomes. The isoenzyme composition of hepatic microsomal carboxylesterase was changed after partial hepatectomy, and marked decreases in RL2 activity and protein content were observed at 4 weeks, at the time of preneoplastic foci induction. Partial hepatectomy alone also resulted in decreased RL2 activity. These findings suggest that RL2 may be involved in regulation of protein kinase C activity by metabolizing its activators at an early stage of hepatocarcinogenesis in rats.

Key words: Carboxylesterase — Phorbol ester — Hepatocarcinogenesis

Non-specific carboxylesterases (EC 3.1.1.1) in rat liver microsomal fractions hydrolyze a number of xenobiotic compounds such as esters, amides and thioesters, and are responsible for the metabolism and detoxication of toxic chemicals from the environment.[1,2] It has been demonstrated that isoenzymes of carboxylesterase have lipase-like activity and are involved in lipid metabolism,[3] and a recent study revealed that certain isoenzymes of carboxylesterase in rat liver also have TPA-hydrolyzing potential.[4] This is of interest, because TPA,[5] a tumor promoter first shown to promote mouse skin carcinogenesis, causes various effects in different tissues in different species by activation of protein kinase C.[5,6] The possible hydrolysis products of TPA, TP, PA or phorbol,[5] are all known to be inactive as promoters[6] and since protein kinase C has been suggested to be involved in carcinogenesis and cell proliferation,[7-10] it is important that changes in carboxylesterase with TPA-hydrolyzing activity during carcinogenesis should be clarified. In a previous paper, we reported the purification and characterization of three isoenzymes of carboxylesterase, RL1, RL2 and RH1, and demonstrated that these three differ from one another in substrate specificity, immunological properties, inducibility by drugs and response to hormones.[11] In the present report, we show that the carboxylesterase isoenzyme, RL2, has a high hydrolyzing activity towards not only TPA but also diacylglycerol, an endogenous activator of protein kinase C, and that it markedly decreases during Solt-Farber (SF) model hepatocarcinogenesis after partial hepatectomy (PH).

MATERIALS AND METHODS

Chemicals [20-^{3}H]12-*O*-Tetradecanoylphorbol-13-acetate ([^{3}H]TPA; specific activity, 12.7 Ci/mmol (469.9 GBq)), was obtained from New England Nuclear through Daiichi Pure Chemicals Co. Ltd., Tokyo. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), phorbol-13-acetate (PA), 12-*O*-tetradecanoylphorbol (TP), phorbol, 1-monooctanoyl-*rac*-glycerol, 1,2-dioctanoyl-*rac*-glycerol and 1,3-dioctanoylglycerol were purchased from Sigma Chemical Co., St. Louis, MO. 1-Oleoyl-2-acetyl-*rac* glycerol was from Calbiochem, La Jolla, CA. Acetanilide and the NEFA C-test for determination of free fatty acids were purchased from Wako Pure Chemicals Inc., Ltd., Tokyo. The glycerol F-kit for determination of glycerol was from Boehringer Mannheim, Mannheim, Germany. Other chemicals were of reagent grade, from commercial sources.

Animals Male Sprague-Dawley rats weighing 170–200 g were used in this study. The animals were group-housed

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[5] Abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TP, 12-*O*-tetradecanoylphorbol; PA, phorbol-13-acetate; SF model, the Solt-Farber model; PH, partial hepatectomy; DEN, diethylnitrosamine; AAF, N-acetylaminofluorene; PAGE, polyacrylamide gel electrophoresis; SRID, single radial immunodiffusion.

in plastic cages with stainless-steel grid tops under conditions of constant temperature (22–24°C) and humidity (50–60%) in the Institute for Animal Experiments of Hirosaki University School of Medicine. Food and water were given *ad libitum.*

Tissue preparations Enzyme altered foci and hyperplastic nodules were induced in rat livers by administration of diethylnitrosamine (DEN) (200 mg/kg of body weight) followed by N-acetylaminofluorene (AAF) (0.02% in diet) plus PH according to the SF model.[12] Rat primary hepatomas were induced by administration of AAF (0.02% in diet) alone to animals for 30 weeks.[13] Normal and hyperplastic nodule-bearing livers were homogenized in 5 vol. of ice-cold 1.15% KCl in a glass homogenizer with a Teflon pestle. Microsomal fractions were obtained as described previously.[11]

Purification of carboxylesterase isoenzymes Carboxylesterase isoenzymes, RL1, RL2, and RH1, were purified from rat liver microsomal fractions, as described previously.[11]

Assay of TPA hydrolysis Hydrolysis of TPA was carried out as described by Mentlein[4] with minor modifications. [^{3}H]TPA was diluted with unlabeled TPA to a radioactivity concentration of 0.1 μCi/nmol and dissolved in acetone to give a concentration of 0.1 nmol/μl. The reaction mixture (0.2 ml) contained 2 nmol of [^{3}H]-TPA, 100 m*M* Tris-HCl buffer (pH 8.0), 0.5% defatted bovine serum albumin, and the enzyme preparation. The reaction was started by addition of the enzyme, carried out at 37°C for 60 min and stopped by the addition of 20 μl of acetic acid. The mixture was evaporated using a centrifugal evaporator (Model RD-41, Yamato Scientific Co. Ltd., Tokyo) and the residue was mixed with 15 nmol of unlabeled TPA, TP, PA or phorbol, respectively. Organic extracts were then applied to silica gel thin layer plates (DC-Alufolien Kieselgel 60 F, Merck, Germany). After double development in dichloromethane/acetone (3:2), the substances were visualized on the basis of their quenching of fluorescence under shortwave UV light (254 nm). The spot areas were scraped off the plate, and the radioactivities were determined using a liquid scintillation counter (Aloka type 1000, Tokyo). The amounts of deacetylation and/or deacylation products formed were calculated from the ratio of the radioactivity in the spots to the amount of unlabeled TPA used in the assay.

Enzyme assays The enzymatic hydrolysis of *p*-nitrophenylacetate was determined spectrophotometrically by measuring the released amount of *p*-nitrophenol at 405 nm in 50 m*M* Tris-HCl buffer (pH 8.0) at 30°C according to the method of Krisch.[14] The enzymatic acetanilide hydrolysis was assayed fluorometrically by measuring the amount of released aniline (ex=282 nm, em=348 nm) at 37°C in 50 m*M* Tris-HCl buffer (pH 8.6) according to the method of Heymann *et al.*[15] The hydrolysis of diacylglycerols was measured both by quantitation of released glycerol using the Glycerol F-kit (Boehringer Mannheim) and by an enzymatic determination of free fatty acids using the NEFA C-test (Wako Chemicals, Tokyo) according to the methods of Mentlein *et al.*[3] with slight modifications. Substrate solutions containing diacylglycerols were prepared by dissolving the glycerides in chloroform/methanol (2/1) together with a 20-fold molar amount of sodium taurocholate and then the solvent was evaporated off with a stream of nitrogen gas. The residue was dissolved in water to yield 10 m*M* solutions. The assay mixtures, containing 2 m*M* diacylglycerol, 40 m*M* taurocholate, and 100 m*M* Tris-HCl buffer (pH 8.0) in a total volume of 500 μl, were incubated for 60 min at 37°C. The reaction was started by addition of the purified enzyme and stopped by adding 2 m*M* sodium dodecyl sulfate, and then the released fatty acids were determined by using the NEFA C-test according to the manufacturer's instructions. The amount of glycerol released from diacylglycerol was estimated enzymatically as described above. Specific activities of carboxylesterases towards *p*-nitrophenylacetate and acetanilide were expressed in μmol and nmol of substrate hydrolyzed per mg protein in 1 min, respectively, under the above specified conditions.

Gel electrophoresis Gel electrophoresis was performed according to the method of Davis,[16] with 7.5% acrylamide slab gels containing 1% Nonidet P-40. After the electrophoresis, gels were stained for esterase activity using 1-naphthylacetate as a substrate according to the method of Mentlein *et al.*[17]

Single radial immunodiffusion (SRID) Immunodiffusion plates were prepared with 0.9% agarose containing 1.0 *M* glycine, pH 7.4, 0.086 *M* sodium chloride, 0.54 *M* glycerol, 0.015 *M* sodium azide, 0.2% Emulgen 913 and 0.5% sodium cholate according to the method of Thomas *et al.*[18] The liver microsomes were solubilized with 2% Emulgen 913 and 10% sodium cholate after being diluted to 10 mg/ml of protein concentration with 1.15% KCl. Monospecific antibody to carboxylesterase RL2 purified from rat liver was raised in a rabbit as previously described.[19] Electrophoretically homogeneous RL2 was used as a standard, and did not cross-react with other antibodies to RH1 and RL1.

RESULTS

Hydrolysis of TPA by the purified carboxylesterase isoenzymes Determination of the TPA-hydrolyzing activities of the purified carboxylesterase isoenzymes, RL1, RL2 and RH1, revealed RL2 to have the highest activity for cleavage of the C-12 ester bond of TPA to form PA, but a relatively low deacylation activity for the C-13 ester bond to form TP (Table I). The other isoenzymes

Table I. Metabolism of 12-*O*-Tetradecanoylphorbol-13-acetate by Carboxylesterase Isoenzymes Purified from Rat Livers

Isoenzyme	Deacylated product formation (nmol/min)		
	PA	TP	Phorbol
RL1	ND	ND	ND
RL2	8.970	0.996	0.023
RH1	0.193	0.007	ND

ND, not detectable.

Table II. Di- and Monoacylglycerol-hydrolyzing Activity of Carboxylesterases

Substrate	Method	Specific activity (μmol/mg protein/min)		
		RL1	RL2	RH1
1,2-Dioctanoylglycerol	A	0.38	2.14	0.23
	B	3.16	4.27	1.83
1,3-Dioctanoylglycerol	A	0.48	1.62	0.26
	B	4.01	3.45	2.13
1-Oleoyl-2-acetylglycerol	A	0.07	0.01	0.01
	B	1.75	1.34	0.05
1-Monooctanoylglycerol	A	0.47	5.18	0.50

A, assayed for glycerol released.
B, assayed by enzymatic determination of free fatty acids.

demonstrated only negligible or much lower activities for cleavage and deacylation.

Diacylglycerols, whose structures are analogous to that of TPA, are also good substrates for the carboxylesterase RL2. The results of hydrolysis of 1,2- and 1,3-diacyl derivatives by carboxylesterases are shown in Table II; RL2 possesses the highest specific activity towards 1,2-dioctanoylglycerol when assayed in terms of glycerol released. Our previous study revealed that RL2 also possesses the highest specific activity towards 1-monooctanoylglycerol (C8:0) among monoacylglycerols with various chain lengths (C2-C18).[20] The activities towards 1,2-diacyl derivatives were slightly higher than that towards 1,3-diacyl derivatives (Table II). The amounts of fatty acid released from 1,2- or 1,3-dioctanoylglycerol were about twice the values for glycerol, suggesting that RL2 releases both 1- and 2- or 3-octanoyl residues. On the other hand, RL1 and RH1 may release only one octanoyl residue, since the amounts of glycerol released were much lower than those of the free fatty acids generated. The amounts of glycerol released by these two isoenzymes from 1-monooctanoylglycerol were similar to the values for 1,2- or 1,3-dioctanoylglycerol,

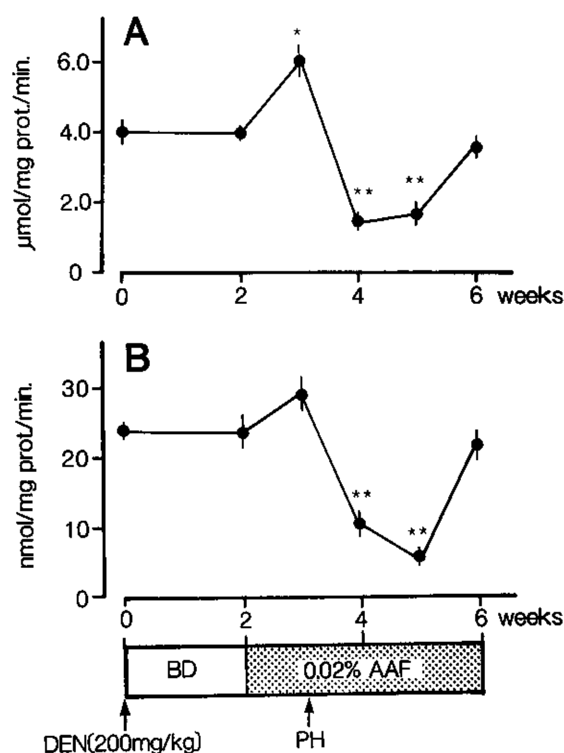

Fig. 1. Changes in activities of microsomal carboxylesterases during rat hepatocarcinogenesis induced by the SF model. A, *p*-Nitrophenylacetate hydrolase activity. B, Acetanilide hydrolase activity. Each value point is the mean±SE of determinations for 6 to 11 animals. *, **, significantly different from control values (at 0 week) with $P<0.01$ and $P<0.001$, respectively. The SF model is shown at the bottom of the figure.

suggesting that RL1 and RH1 act on 2- or 3-octanoyl in preference to 1-octanoyl residues. Furthermore, RL2 as well as RL1 demonstrated hydrolyzing activity towards 1-oleoyl-2-acetylglycerol, which is often used in the study of the protein kinase C as an analogue of its activator, TPA.[7]

Changes in activities of carboxylesterases during hepatocarcinogenesis Changes in activities of liver microsomal carboxylesterases during hepatocarcinogenesis by the SF model are illustrated graphically in Fig. 1. Since *p*-nitrophenylacetate is hydrolyzed by most of the known serine hydrolases, total carboxylesterase activity was measured using it as the substrate. On the other hand, acetanilide is a more specific substrate for carboxylesterase RL2.[11] The total activity was significantly increased 3 weeks subsequent to the initial DEN injection (after one week of AAF administration) (Fig. 1A), while the acetanilide-hydrolyzing activity also tended to increase, but not significantly (Fig. 1B). At week 4 after

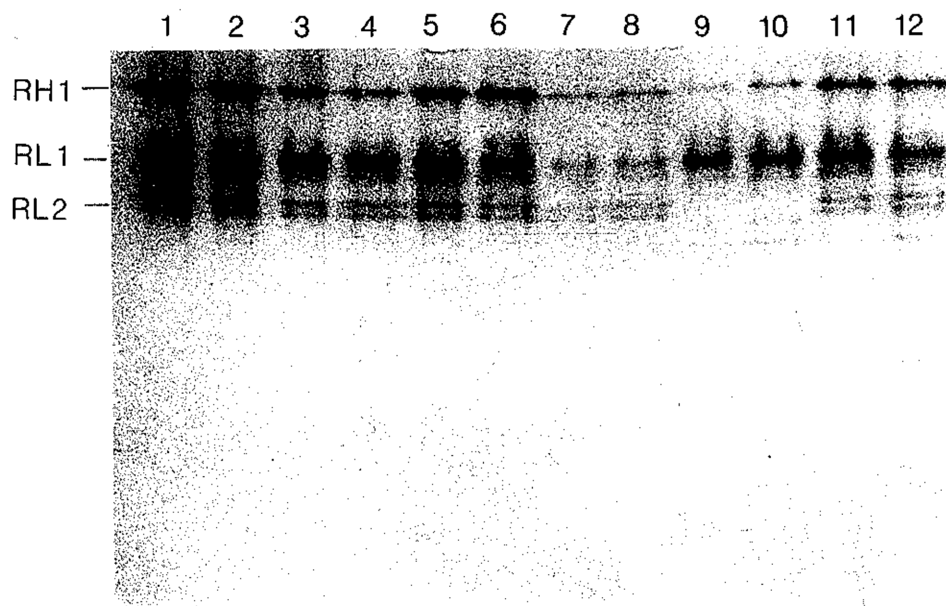

Fig. 2. Changes in isoenzymic pattern of carboxylesterase during rat hepatocarcinogenesis. Detergent-solubilized microsomal preparations (7.5 μg each) from individual livers were subjected to PAGE followed by staining for esterase activity. Lanes 1 and 2, normal livers; lanes 3 and 4, livers at 2 weeks after DEN injection; lanes 5 and 6, resected livers at 3 weeks; lanes 7 and 8, livers 1 week after PH; lanes 9 and 10, livers 2 weeks after PH; lanes 11 and 12, livers 3 weeks after PH.

Table III. Changes in Content of Carboxylesterase during Rat Hepatocarcinogenesis by the Solt-Farber Model

	RL2 content (μg/mg protein)
Normal liver	58.0±2.5
2 weeks after DEN inj.	52.3±8.4
Resected liver	68.0±3.7
1 week after PH	8.3±1.8**
2 weeks after PH	8.1±3.5**
3 weeks after PH	53.5±11.7

Immunochemical quantitation of carboxylesterase isoenzyme RL2 was conducted by the method of single radial immunodiffusion. Each value is the mean±SE of data for four animals.
** Significantly different from normal with $P<0.001$.

Table IV. Changes in Content of Carboxylesterase RL2 in Regenerating Rat Livers

	RL2 content (μg/mg protein)
Normal liver	64.1±2.4
2 days after PH	37.2±0.6**
1 week after PH	32.7±4.9*
2 weeks after PH	41.5±14.6
3 weeks after PH	50.4±4.6

Immunochemical quantitation of carboxylesterase isozyme RL2 was conducted by the method of single radial immunodiffusion analysis. Each value is the mean±SE of data for three animals.
*, ** Significantly different from the normal value with $P<0.01$ and $P<0.001$, respectively.

the DEN injection (one week after PH), both the total and acetanilide-hydrolyzing activities were markedly decreased. The total activity returned to the normal level by week 6, while the acetanilide-hydrolyzing activity continued to decrease till week 5 and then rapidly returned to the normal level by week 6.

In the normal liver, three distinct carboxylesterase activity bands were detected by PAGE of liver microsomal proteins followed by activity staining with 1-naphthylacetate (Fig. 2). Carboxylesterase RL2 showed two activity bands with similar mobilities (Fig. 2). This might be due to charge microheterogeneity of this enzyme, since the purified RL2 showed a single band on PAGE in the presence of sodium dodecyl sulfate.[11] In accordance with the changes in the activity shown in Fig. 1, all the bands of the three isoenzymes were re-

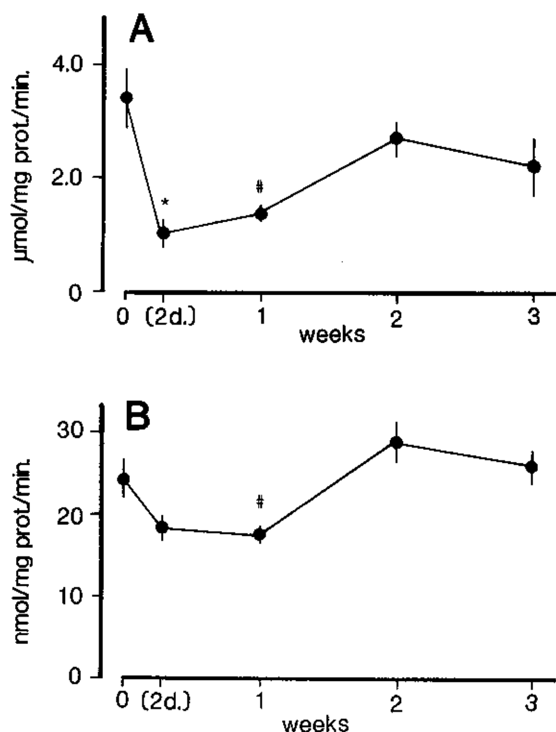

Fig. 3. Changes in activities of carboxylesterases in regenerating rat liver microsomes. A, *p*-Nitrophenylacetate hydrolase activity. B, Acetanilide hydrolase activity. Each value is the mean±SE of determinations for 3 to 4 animals. *, #, significantly different from control values (at 0 week) with $P<0.01$ and $P<0.05$, respectively.

duced at week 4 (lanes 7 and 8, Fig. 2), and in particular the two RL2 bands both completely disappeared at week 5 (lanes 9 and 10). In contrast, RL1 had recovered to almost normal levels by week 5. The RL2 bands were again present at week 6 but were still reduced as compared to the normal level.

The protein content of RL2 in these preparations was quantified by SRID using the monospecific antibody. As is evident in Table III, the content of RL2 dramatically decreased one week after PH, remaining at a similar low level for a further week before returning almost to normal by week 3.

The effects of PH on carboxylesterase isoenzymes The activities of carboxylesterase in regenerating livers after PH were examined as a control for the SF model (Fig. 3). Both the total and acetanilide-hydrolyzing activities decreased after 2 days, and gradually returned to the normal levels by 2 weeks after PH. As shown in Table IV, the content of RL2 measured by SRID was 51% of normal at one week, but at 3 weeks it was 79% of the normal value.

Table V. Carboxylesterase Activities in Primary Hepatomas and Yoshida Ascites Hepatomas

	p-Nitrophenylacetate hydrolase activity[a]	Acetanilide hydrolase activity[b]
Normal liver	4.01±0.35	23.60±1.10
Hepatoma AAF-induced	1.36±0.38	14.42±1.00
Transplantable hepatoma		
AH 13	<0.05	<1.0
AH 130	<0.05	<1.0

a) Expressed as μmol/mg prot./min.
b) Expressed as nmol/mg prot./min.

Carboxylesterase activities in primary hepatomas and Yoshida ascites hepatomas Carboxylesterase activities in primary hepatomas induced by AAF and transplantable Yoshida ascites hepatomas are shown in Table V. In primary hepatomas, hydrolase activities towards both *p*-nitrophenylacetate and acetanilide were decreased, to 34% and 61% of the normal values, respectively. In transplantable Yoshida ascites hepatomas, AH13 and AH130, they were hardly detectable.

DISCUSSION

Mentlein indicated that rat liver homogenate or cell fractions can deacylate TPA mainly to form PA, two esterase isoenzymes purified from rat liver with pI 5.2 and pI 5.6, respectively, being largely responsible.[4] It is not clear whether or not RL2 is identical with one of these esterases, although there is a clear similarity between RL2 with a pI value of 5.5 and the hydrolase with pI 5.6 purified by Mentlein. TPA hydrolases have also been isolated from murine liver cytosol by Shoyab *et al.*,[21] while Berry *et al.*[5] described activity located in the microsomes of mouse liver. It is well known that there are species differences regarding carboxylesterase; i.e., in isoenzyme pattern, substrate specificity and other properties.[20] TPA-hydrolyzing activities of purified hepatic carboxylesterases from several mammalian species showed a variety of rates of metabolic conversion (our unpublished results) similar to that evident for the different rat liver isoenzymes in Table I. Of the isoenzymes purified so far from mammalian species, RL2 from rat liver shows the highest TPA hydrolytic activity, PA mainly being formed (see Table I). In the present study, purified carboxylesterase RL2 also showed significant activity towards 1-oleoyl-2-*rac*-acetyl-glycerol (Table II), which has often been used as an activator of protein kinase C.[7] The results thus suggest that RL2 might prevent the accumulation of diacylglycerols or other TPA-like activators in rat liver by converting them to

inactive forms and thereby indirectly block the activation of protein kinase C. This would affect the series of cellular responses induced by protein kinase C and perhaps interfere with some stage of hepatocarcinogenesis. To our knowledge, however, there has been no report showing that TPA is effective as a promoter for rat hepatocarcinogenesis.

The activation of protein kinase C is dependent on the stereostructure of diacylglycerols, and only 1,2-diacyl-*sn*-glycerols can activate it in the presence of Ca^{2+} ion and phospholipids.[7] On the other hand, hydrolysis of diacylglycerol by carboxylesterase RL2 was not stereospecific; i.e. RL2 hydrolyzes not only 1,2-diacylglycerols but also 1,3-diacylglycerols, as is evident from the results in Table II. Therefore, possible functions of carboxylesterase RL2 might include diminishing the concentration of cytotoxic diacylglycerols or monoacylglycerols produced from phospholipids by phospholipase C rather than acting specifically on 1,2-diacylglycerols involved in the activation of protein kinase C.

In the SF model, RL2 carboxylesterase was decreased markedly at week 4 in both activity and content (Fig. 1, Table III). The decrease of this isoenzyme was confirmed by PAGE (Fig. 2). This indicates a diminished ability to metabolize diacylglycerols. Since RL2 was decreased to 14% of the normal value at 4 weeks after DEN injection in the SF model (at 2 weeks after PH) (Table III), the results were not solely due to the effects of PH, which alone caused a decrease in the content of RL2 to 50% of the normal value (Table IV). This latter finding of lowered metabolizing capacity for diacylglycerols, suggests that protein kinase C may be activated in regenerating rat liver. However, the decrease in RL2 in the SF model appears clearly linked not only to PH, but also to the generation of preneoplastic lesions.

Previously, Kaneko *et al.*[22] reported an isoenzyme of carboxylesterase termed microsomal butyrylesterase (L-1), which was hardly observed in the normal adult liver but was detectable in fetal and neonatal livers. It was increased in the regenerating liver after PH and in the early stage of hepatocarcinogenesis by 3′-methyl-diaminoazobenzene, and was also induced by phenobarbital.[23] This butyrylesterase L-1 may be identical to the isoenzyme of carboxylesterase, designated RL3 by ourselves, which was purified from clofibrate-treated rat livers and could not be detected in normal liver (our unpublished results). This isoenzyme, RL3, could also be differentiated from RL2 on PAGE (results not shown).

The activation mechanism of protein kinase C has been studied in many tissues. The present study, in revealing alteration of RL2 metabolism of activators of protein kinase C during hepatocarcinogenesis, suggests a possible role in early-stage hepatocarcinogenesis.

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