Hepatic Protein Kinase-C and Protein Phosphatase Type-2A in the Fetal Rat

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ABSTRACT. The Ca^{2+} - and phospholipid-dependent protein kinase, protein kinase-C (PK-C), was studied in fetal rat liver between d 17 and 21 of gestation. Initial studies showed that rat liver, membrane-associated PK-C could be detected as a protein of $M_r = 80\,000$ using a polyclonal rat brain PK-C antiserum. Fetal hepatic membrane-bound PK-C activity rose as gestation progressed with adult levels (21 ± 3 pmol/min/mg protein) being attained by term. Although fasting for 48 h led to PK-C activation in adult livers, fetal hepatic PK-C was not activated by 48 h of maternal fasting. Membrane-associated protein phosphatase activity that might reverse PK-C action was also studied. PK-C sites in casein (an artificial PK-C substrate) were selectively dephosphorylated by a membrane-associated, poly-cation-stimulated protein phosphatase. This activity, thus classified as protein phosphatase type-2A, was constitutively expressed in fetal liver membranes from 17–21 d gestation. We have previously reported that the other major hepatic protein phosphatase, protein phosphatase type-1, also is constitutively expressed during the later stage of gestation. Taken together with the results of our present study, our data indicate that PK-C-dependent phosphorylation in fetal liver probably increases with advancing gestation. (*Pediatr Res* 27: 599–603, 1990)

Abbreviations

HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
I-2, inhibitor-2
MOPS, 3[*N*-morpholino]propanesulfonic acid
PK-C, protein kinase-C
PP-1, protein phosphatase type-1
PP-2A, protein phosphatase type-2A
TCA, trichloroacetic acid

PK-C, first isolated from rat brain as a protease-activated enzyme (1), is now known to represent a family of Ca^{2+} - and phospholipid-dependent protein kinases that are ubiquitous in mammalian tissues (2, 3). These enzymes are activated by the production of diacylglycerol via inositol phospholipid hydrolysis. They are, therefore, likely participants in the transmembrane signalling for a variety of hormones. Numerous substrates for PK-C, including enzymes of intermediary metabolism, contractile and cytoskeletal proteins, ribosomal protein S6, oncogene products, and hormone receptors have been found (2). The variety of these substrates presumably explains the pleiotropic

role of this enzyme in regulating metabolic processes as well as cell growth. In addition, the powerful tumor promoters, phorbol esters, are known to exert their effects through the direct activation of PK-C (3). Given this background, it seems reasonable to speculate that, in fetal liver, PK-C might play a role in both growth and metabolic differentiation.

Although considerable progress has been made in characterizing substrates for PK-C, little is known about the protein phosphatases that are active against PK-C sites. The posttranslational modification of proteins by phosphorylation is a rapidly reversible process (4). This reversibility, via the action of specific protein phosphatases, is required for the regulatory function of protein phosphorylation. Protein phosphatases can be classified on the basis of substrate specificity and modulation by various agents. The major protein phosphatases in rat liver are PP-1 and PP-2A (4). PP-1 is distinguished by its sensitivity to the phosphatase regulatory protein, I-2, whereas PP-2A is characterized by poly-cation activation (4). We have reported previously that hepatic PP-1 activity is present at relatively constant levels between d 17 of gestation and term in the fetal rat (5). Given that active PK-C is membrane-associated (6, 7), to study further the reversal of PK-C action by protein phosphatases, we also examine fetal hepatic membrane-associated PP-2A activity in our present studies.

MATERIALS AND METHODS

Pregnant Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were delivered by cesarean section on d 17–21 of gestation. Control dams (20 litters) were fed standard laboratory food *ad libitum*. For experimental groups, mothers were fasted for 24 h (three litters) or 48 h (four litters) before delivery on d 20 or 21, respectively. Fasted animals were given free access to water. Cesarean section was performed under pentobarbital anesthesia (50 mg/kg, intraperitoneally). With the placenta adherent to the uterus, the fetuses were exsanguinated via an axillary incision. Their livers were removed, flash frozen in liquid nitrogen, and stored at -70°C until use. Livers from 125–150 g male rats, fed *ad libitum* ($n = 6$) or fasted for 48 h ($n = 6$), were also studied.

Membrane and cytosol preparations were made from adult livers and pooled fetal livers (one preparation per litter) by homogenizing rat liver at a ratio of 1 g liver per 10 mL buffer (50 mM HEPES, pH 7.4, 0.25 M sucrose, 5 mM EDTA, 250 $\mu\text{g/mL}$ phenylmethyl-sulfonyl fluoride, 10 $\mu\text{g/mL}$ leupeptin, 1 TIU/mL aprotinin). The homogenate was centrifuged at $4000 \times g$ for 15 min. The supernatant was centrifuged at $40\,000 \times g$ for 20 min. The supernatant from this centrifugation was considered cytosol. The resulting membrane pellet was resuspended in homogenizing buffer (1 mL per g of liver) and recentrifuged at $40\,000 \times g$ for 20 min. The pellet was resuspended, this time in 50 mM HEPES, pH 7.4 (1 mL per g of liver) and centrifuged at $40\,000 \times g$ for 20 min. The pelleted membranes were resuspended to a protein content of 10 mg/mL. The final membrane

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suspension was stored at -70°C until use. Membrane protein content was determined using bicinchoninic acid (BCA, Pierce Chemical Co., Rockford, IL) with BSA as standard. Yields of membrane and cytosolic protein per unit wet wt were similar for the various gestational ages and experimental (adult and fetal) groups.

PK-C activity was measured in the presence of Ca^{2+} as histone kinase activity (4) and was defined as activity measured in the presence of phosphatidylserine and dioleoin minus the activity measured in their absence.

PK-C was partially purified from adult rat liver membranes freshly prepared (6 mL of a membrane suspension containing 60 mg protein) and extracted with 6 mL 50 mM HEPES, pH 7.4, 2% (vol/vol) NP40 detergent containing protease inhibitors (as above). The extract was clarified by centrifugation at $40\,000 \times g$ for 30 min and batch-adsorbed to 15 mL DEAE-Sephacel CL-6B. The gel was placed in a column over 3 mL of fresh gel and washed with three column volumes of 20 mM HEPES, pH 7.4, 1 mM DTT, 50 mM NaCl, 1% NP40. PK-C activity was eluted as a single peak with a linear concentration gradient from 0.05 M to 0.4 M NaCl in the same buffer. [Partial purification of PK-C using ion exchange chromatography is discussed further in Results (Fig. 1)]. The active fractions were pooled and frozen in aliquots at -70°C until use. The histone kinase activity of this preparation was 5 pmol/min/mL in the presence of 1 mM EDTA, 49 pmol/min/mL in the presence of 2 mM Ca^{2+} , and 131 pmol/min/mL in the presence of Ca^{2+} plus lipid micelles.

Substrate for protein phosphatase assays was prepared by incubating 20 mM MOPS, pH 7.5, 20 mM Mg acetate, 1.2 mM CaCl_2 , 1 mM ATP in 1 mL containing 0.5 mg phosphatidylserine, 20 μg dioleoin, 50 μCi [$\gamma\text{-}^{32}\text{P}$]ATP, 0.1 mL partially purified PK-C, and 1 mg casein at 30°C for 16 h. The casein was prepared by prior TCA-precipitation (8). The phosphorylation reaction was stopped by adding 0.2 mL 100% TCA. The precipitated substrate was collected by centrifugation, washed two times with 20% TCA, redissolved in 0.1 mL 1 N NaOH and diluted to 10 μM with assay buffer (20 mM MOPS, pH 7.5, 20 mM glucose, 1 mM DTT, 1 mM theophylline, 1 mg/mL BSA).

Casein phosphatase activity was measured as the release of ^{32}P from the above substrate. Sample (20 μL) and substrate (20 μL) were diluted in assay buffer (20 mM MOPS, pH 7.5, 20 mM glucose, 15 mM 2-mercaptoethanol, 1 mM theophylline, 1 mg/

mL BSA) and incubated for 10 min at 30°C . The reaction was stopped by the addition of 0.06 mL 25 mg/mL BSA and 0.9 mL 20% TCA. After centrifugation in a bench-top microfuge for 3 min, 0.9 mL of the supernatant was transferred to a scintillation vial for determination of released ^{32}P . Results are corrected for TCA-soluble ^{32}P present in the substrate; this did not exceed 10% of total ^{32}P .

Phosphorylase phosphatase activity was measured as previously described (9) with and without prior activation by Co^{2+} plus trypsin. I-2 was purified from rabbit skeletal muscle (9).

Western immunoblots used polyclonal antibodies raised in a goat against heterogeneous rat brain PK-C (10). These antibodies react with two of the three isozymes that are distinguished by hydroxylapatite column chromatography of rat brain extracts. A previously described method for Western immunoblotting (9), which uses ^{125}I -protein A and autoradiography for detection, was modified for the use of goat primary antibody.

RESULTS

In preliminary experiments, the distribution of hepatic PK-C activity between soluble and particulate fractions was studied. In three separate preparations from three adult rat livers, 60, 47, and 76% of total activity was recovered in the membrane detergent extract. In these preparations, as was found in subsequent studies, membrane-associated sp act was approximately 10-fold higher than sp act in the soluble fraction.

Western immunoblotting was used to identify hepatic PK-C, which has previously been purified as a $M_r = 64\,000$ protein (11). Gradient ion exchange chromatography of a membrane extract was done (as described in Methods) to compare detection of immunoreactive proteins with PK-C activity. Results (Fig. 1) showed that the peak of PK-C activity, defined as lipid-micelle-dependent histone kinase activity, coincided with an immunoreactive protein of $M_r = 80\,000$. Several crossreactive proteins without histone kinase activity, with M_r of $\sim 150\,000$, 62 000, and 44 000, were detected in other regions of the chromatogram (not shown) and were unassociated with kinase activity. Detection of all immunoreactive proteins required anti-PK-C primary antibody. Thus, these experiments indicate that rat liver membrane-associated PK-C activity resides in a protein with $M_r = 80\,000$. Immunoreactive proteins without histone kinase activity are presumed to be unrelated to PK-C.

Hepatic membrane-associated PK-C sp act increased with advancing gestational age (Fig. 2), reaching adult levels by d 21. Membrane-associated PK-C activation by varying concentrations of mixed lipid micelles was similar in term fetal and adult liver (data not shown). PK-C specific activity in cytosol (Fig. 2) was approximately $1/10$ that in membrane fractions. Soluble sp act was unchanged as gestation proceeded with fetal activities approximating those in adult liver.

Fasting for 48 h in the adult rat produced a consistent increase in membrane-associated activity (Fig. 2). No concomitant change in cytosolic activity was observed. In contrast, maternal fasting for 48 h did not affect fetal hepatic membrane-associated or cytosolic activity. We have previously characterized the fasted pregnant rat as a model of intrauterine growth retardation in our own laboratory. As in previous studies (5, 12), maternal fasting for 48 h led to fetal hypoinsulinemia with impaired fetal growth ($\sim 10\%$ decrease in fetal carcass wt and $\sim 25\%$ decrease in fetal liver wt). Impaired fetal hepatic growth in this model is associated with an increase in DNA content, but no change in protein content (5), indicating a diminution in cell size.

Western immunoblotting of unfractionated fetal and adult liver membrane preparations was done (data not shown) but yielded inconclusive results. A protein with $M_r = 80\,000$ was seen at consistent levels throughout gestation (in contrast to changing PK-C activity). The levels of this immunoreactive protein also did not increase in livers from fasted adult rats. Heterogeneity in the proteins detected (as noted in the ion-

Fig. 1. Purification of rat liver membrane-associated PK-C. A 1% NP40 extract of rat liver membranes was adsorbed to and eluted from DEAE-Sephacel CL-6B as described in Methods. Protein kinase activity was measured in the presence of calcium alone (diamonds) or calcium plus lipid micelles (circles). The dashed line depicts the NaCl gradient. The autoradiogram on the right shows results of a Western immunoblot using antirat brain PK-C antiserum. The numbers above the lanes correspond to the fractions from the chromatogram. The arrow to the left of the autoradiogram designates an immunoreactive protein with $M_r = 80\,000$.

Fig. 2. PK-C activity in fetal and adult rat liver. Activities in membrane (upper panel, circles) and cytosolic (lower panel, squares) fractions are shown. Fetal rats with gestational age 17 to 21 d were studied. Open symbols denote fetuses of mothers fasted for 48 h or, for adult rats, animals killed after a 48-h fast. The numbers in parentheses represent activities for two measurements that were well above the range for the other animals.

exchange chromatogram) and an unexpectedly high level of $M_r = 80\,000$ immunoreactive protein raised doubts as to the identity of this protein with authentic PK-C. It was concluded that detection of proteins unrelated to PK-C interfered with estimates of PK-C content in unfractionated membranes.

To study the dephosphorylation of PK-C sites, casein was phosphorylated by PK-C to a concentration of $10\text{--}15\ \mu\text{M}$ ^{32}P . Initial studies showed that phosphatase activity against this substrate was present in a 1% Triton X-100 extract of rat liver membranes. Dephosphorylation of the substrate at a final concentration of $5\ \mu\text{M}$ was linear versus time and enzyme concentration with hydrolysis of up to 25% of the total substrate. Measurements, therefore, were made on samples diluted sufficiently to result in substrate hydrolysis in the linear range of the assay.

The pH optimum for dephosphorylation of casein phosphorylated by PK-C was determined using cacodylate (pH 5.5, 6.0, 6.5, 7.0) and HEPES (pH 7.0, 7.5, 8.0) buffers. Activity was maximal at pH 7.0 (results not shown). Addition of a variety of agents that are known to modify protein phosphatases resulted in the following changes in casein phosphatase activity (control = 100%): Ca^{2+} (2 mM), 108%; Mg^{2+} (2 mM), 118%; Co^{2+} (0.4 mM), 96%; and ATP (2 mM), 95%. Spermine (1 mM) activated membrane-associated phosphatase activity 2- to 5-fold.

The kinetics of casein dephosphorylation showed a K_m for casein phosphorylated by PK-C less than $6\ \mu\text{M}$ in three replicate experiments on three membrane preparations. Kinetic analysis in the presence of the poly-cation spermine showed a 50–60% decrease in K_m with no change in $V_{\max}$.

No inhibition of membrane-associated casein phosphatase activity was seen with addition of the PP-1 regulator, I-2, at concentrations up to 4000 U/mL. (One unit of I-2 activity is defined as the amount that can inhibit a standard PP-1 preparation with 1.5 nmol/min/mL activity by 50%). Treatment of a membrane extract with Co^{2+} plus trypsin did not activate casein phosphatase activity. By contrast, phosphorylase phosphatase activity in membrane extracts (measured at 15 mM phosphorylase a) was stimulated 2.5- to 5-fold by Co^{2+} plus trypsin treatment. Casein phosphatase activity in fetal liver membrane ex-

tracts behaved similarly to that obtained from adult liver. Spermine activated casein dephosphorylation, whereas Co^{2+} plus trypsin treatment was ineffective.

To further characterize the casein phosphatase activity, 25 mL of an adult liver membrane preparation containing approximately 500 mg of protein was extracted for 30 min at 4°C with 25 mL 50 mM HEPES, pH 7.4, 2% Triton X-100. The extract was clarified by centrifugation at $40\,000 \times g$ for 30 min and adsorbed to DEAE-Sepharose CL-6B (20 mL, equilibrated in 20 mM MOPS, 1 mM DTT) by mixing for 3 h at 4°C . The gel was applied to a column and washed with the same buffer containing 0.05 M NaCl until absorbance at 280 nm returned to baseline. The phosphatase activity was eluted with a linear gradient of NaCl (0.05 to 0.5 M) in a total volume of 400 mL. Flow rate was 30 mL/h and 5 mL fractions were collected. Protein phosphatase activity was measured without additions or in the presence of 1 mM spermine.

Casein phosphatase activity (measured without the addition of spermine) eluted as a single peak (Fig. 3). The addition of spermine increased activity in the peak approximately 2.5- to 3-fold. A second peak of activity was also measured in the presence of spermine. It eluted just before the constitutive activity and was activated approximately 10-fold by spermine. A minor peak of activity eluting at approximately 0.3 M NaCl was not activated by spermine.

The membrane-associated casein phosphatase activity was measured in livers from control and growth-retarded fetuses (Fig. 4). Activity was present at a nearly constant level from 17 to 21 d and was not affected by maternal fasting.

DISCUSSION

Studies on the expression of PK-C must take into account the heterogeneity of the Ca^{2+} - and phospholipid-dependent protein kinases. At least seven such protein kinases exist (3). Differences in their structure presumably confer differences in function, based perhaps on substrate specificity and mechanisms of regulation. In addition, the issue of subcellular localization must be taken into account when ascribing specific effects to this family of kinases.

The liver is unusual in that a disproportionately high level of PK-C activity remains membrane-associated after homogenization in the presence of 5 mM EDTA. Azhar *et al.* (11) found that approximately 85% of hepatic activity was membrane-associated if membranes were prepared in the absence of chela-

Fig. 3. Partial purification of casein phosphatase by DEAE-Sepharose CL-6B chromatography. A rat liver membrane extract was adsorbed to and eluted from DEAE-Sepharose CL-6B as described in Results. Dephosphorylation of casein phosphorylated by PK-C was measured without additions (circles) and in the presence of 1 mM spermine (squares). The activity in fraction 5 represents activity present in a large peak of protein eluted by buffer containing 0.05 M NaCl. The dashed line depicts the NaCl gradient.

Fig. 4. Protein phosphatase activity in fetal liver membranes. Protein phosphatase activity, identified as type 2A based on the experiment shown in Figure 4, was measured in liver membrane extracts from normal fetuses (closed circles) and fetuses of mothers fasted for 48 h before delivery (open circles).

tors. For the purpose of purifying the rat hepatic PK-C, they adopted the strategy of extracting membrane-associated activity with chelators in increased concentrations (10 mM EDTA, 10 mM EGTA). They succeeded in purifying three isozymes that could be separated on hydroxylapatite columns and that had $M_r = 64\ 000$ on denaturing polyacrylamide gels. However, the deduced amino acid sequence of the various PK-C isozymes indicates a mol wt of approximately 80 000.

In our studies, hepatic membrane-associated PK-C, when partially purified by ion-exchange chromatography, was detected as an immunoreactive protein with $M_r = 80\ 000$. The predominant forms of PK-C in rat liver, based on hydroxylapatite chromatography, are C-II and C-III (11). Both forms are recognized by the antibodies we used (13). Immunoblot analysis of various unfractionated extracts from rat tissue using these antibodies (10) detected immunoreactive bands with $M_r = 80\ 000$. This protein was of low abundance in unfractionated rat liver extracts (10). It appears that we were able to enrich for immunoreactive PK-C by using membrane extracts. The discrepancy between the reported size of the purified rat liver enzyme (11) and that of the immunoreactive PK-C in membrane extracts presumably is due to the occurrence of limited proteolysis during purification.

As noted above, liver is unusual in the high proportion of PK-C that is recovered in the membrane fraction after standard extraction procedures. In a variety of cells in culture, activation of PK-C is manifested as translocation from soluble to membrane-associated fractions (6, 7). However, in some systems, the biologic effects and translocation of PK-C do not correlate (14). Since a reciprocal relationship between soluble and particulate PK-C activities was not observed in our study, activity measurements reported herein should be interpreted as indicating PK-C content rather than *in vivo* activity.

Investigation into a possible regulatory role for PK-C in fetal hepatic development requires study of the dephosphorylation of PK-C sites, inasmuch as reversibility is critical to regulation by protein phosphorylation. Because hepatic PK-C is largely membrane-bound, and because activation of the enzyme generally is associated with translocation to the particulate fraction, the membrane fraction was used as the source of phosphatase activity. Casein was chosen as a substrate for PK-C site phosphatase measurements because it is a selective substrate for PK-C (15). Our studies indicate that PK-C sites in casein are preferentially dephosphorylated by a PP-2A that is present in membrane extracts. This is based on a number of characteristics. Most importantly, the casein phosphatase activity in membrane extracts after fractionation by ion-exchange chromatography was stimulated by spermine (16). In addition, the enzyme exhibited a neutral pH optimum, unlike acid and alkaline phosphatases.

Its activity was not sensitive to the type-1 protein phosphatase regulator, I-2 (17). Lack of identity with PP-1 was indicated further by the lack of activation after Co^{2+} plus trypsin, a treatment that did activate membrane-associated phosphorylase phosphatase activity and that potentially activates latent forms of both rabbit skeletal muscle (18, 19) and rat liver (5) PP-1.

These results are consistent with those of Jakes and Schlender (20), who recently reported that histone H1 phosphorylated by PK-C from rat brain was a selective substrate for the assay of PP-2A (from bovine heart or rat liver nuclei), even in the presence of rabbit skeletal muscle PP-1. However, little information is available on the dephosphorylation of physiologic PK-C substrates. PK-C phosphorylation of muscle glycogen synthase is largely confined to sites 1a and 2 (15), which are substrates for PP-1 and PP-2A (21). In contrast, ribosomal protein S6, a regulator of protein synthesis that may be activated by PK-C phosphorylation (22), is dephosphorylated selectively by PP-1 (23). However, it should be noted that the precise analysis of PK-C phosphorylation sites necessary for correlation with phosphatase specificity has not yet been done. The finding that dephosphorylation of casein, a nonphysiologic PK-C substrate, is catalyzed by PP-2A, does not preclude involvement of other phosphatases in the reversal of PK-C action. The other major serine/threonine protein phosphatase in rat liver is PP-1. We have recently reported that hepatic PP-1 activity, measured with phosphorylase α , is expressed at constant levels through late gestation in the rat (5). Similarly, emphasis in our study on membrane-associated activity does not preclude involvement of soluble protein phosphatases. However, recent data (24) indicate that PP-1 is colocalized with its substrates and kinases for those substrates via the action of "targeting" subunit. Such data are not yet available for PP-2A, but do provide a rationale for the measurement of kinase and phosphatase activities in the same subcellular fraction.

In summary, hepatic membrane-associated PK-C activity increases through the later stages of gestation, reaching adult levels at term. Furthermore, PK-C-dependent protein phosphorylation probably increases as term approaches, given the constitutive expression of fetal hepatic membrane-associated PP-2A activity. Extension of our results to an understanding of the role of PK-C in regulation of fetal hepatic growth requires knowledge of the physiologic substrates for this kinase in fetal rat liver membranes. Potential substrates are numerous, and understanding the role of this enzyme in fetal development will require further study of its regulation and actions at the molecular level.

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