Vol. 120, No. 2, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 30, 1984 Pages 593-601

RETINOID-BINDING PROTEINS ARE PHOSPHORYLATED IN VITRO BY SOLUBLE Ca^{+2}- AND PHOSPHATIDYLSERINE-DEPENDENT PROTEIN KINASE FROM MOUSE BRAIN

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Received March 23, 1984

SUMMARY: Cellular retinol-binding protein (cRBP) and cellular retinoic acid-binding protein (cRABP) were purified from calf liver and uterus, respectively. Soluble Ca^{+2}- phosphatidylserine-dependent protein kinase-C (PK-C) derived from mouse brain was capable of phosphorylating both of these apoproteins in vitro as determined by the phosphocellulose binding assay. The K_m value was determined to be 6.2 μM for apo-cRBP and 5.1 μM for apo-cRABP. In contrast, the K_m value for the histone III-S fraction was estimated to be 10.8 μM; the K_m values for ATP in the presence of apo-cRBP and apo-cRABP were 12.4 μM and 2.6 μM, respectively. Specificity of phosphorylation of the retinoid-binding proteins was confirmed by polyacrylamide gel electrophoresis and subsequent autoradiography of the assay mixture as well as by a concentration-dependent, Ca^{+2}, and phosphatidylserine sensitivity of the phosphorylation of both apo-cRBP and apo-cRABP. Inhibition of PK-C activity by holo-cRBP and holo-cRABP was also observed. Thus, phosphorylation of both of the retinoid-binding proteins may play an important modulating role in i) the ability of retinoids to function as antipromoters in chemically-induced tumorigenesis and ii) the control of physiological aspects of retinoid action in normal and retrodifferentiated cells.

The mechanism of chemical carcinogenesis is currently characterized as a multistep process involving at least two phases, initiation and promotion (1) which provides a useful model for defining these aspects of tumorigenesis and evaluating chemopreventive agents is the mouse skin system. Initiation in this system is achieved by application of a subcarcinogenic dose of a chemical (such as dimethylbenz[a]anthracene); tumors are elicited by repeated applications of a noncarcinogenic compound, the promoter to initiated skin (2).

Abbreviations used: TPA, 12-O-tetradecanoylphorbol-13-acetate; PK-C, Ca^{+2}-phosphatidylserine-dependent protein kinase C; cRBP, cellular retinol-binding protein; cRABP, cellular retinoic acid-binding protein; RBP's, retinoid-binding proteins; PAG, polyacrylamide gel; SDS-PAG, sodium dodecylsulfate-polyacrylamide gel; PS, phosphatidylserine.

0006-291X/84 $1.50

Particularly effective as a promoter is the phorbol ester, 12-O-tetracanoylphorbol-13-acetate (TPA) . The tumorigenic capacity of phorbol esters correlates with the ability of this compound-class to bind to plasma membrane-associated Ca^{+2}- phosphatidylserine-dependent protein kinase-C (PK-C), the apparent phorbol ester receptor, and with activation of PK-C-dependent phosphorylation of numerous cellular proteins (3-5). Some retinoids, such as retinol and especially retinoic acid, have been shown to be effective antipromoters, inhibiting phorbol ester-induced tumorigenesis in the mouse epidermal system when applied in close time proximity with TPA (6-8). It has been suggested that the antipromoter activity as well as normal physiological functions of retinoids may be mediated by their binding proteins, cellular retinol-binding protein (cRBP) and cellular retinoic acid-binding protein (cRABP), since these proteins confer ligand specificity for plasma membrane, endoplasmic reticulum, and nuclei (9-13). Thus, we questioned whether there is some interaction between retinoid-binding proteins (RBP's), their ligands, and the TPA receptor, PK-C, i.e., whether RBP's might act as substrates for PK-C. We present evidence here that at least two of the retinoid-binding proteins, cRBP and cRABP, can act as substrates for PK-C and suggest that this phenomenon may be intimately linked to tumorigenesis.

MATERIALS AND METHODS

Cellular Retinoid-Binding Proteins -- cRBP and cRABP were purified from calf liver and calf uterus, respectively, as previously described (14,15). cRBP and cRABP were purified to approximately 92% and 82% of homogeneity, respectively. These values were determined by polyacrylamide gel (PAG) electrophoresis assay of the proteins subsequent to exposure to UV. light (16). M_r values of 14.6 Kd for each protein, a binding ratio of 1:1 for their respective ligands, and Kd values for retinoid-binding protein-ligand interactions previously determined were used in computing the degree of purification of the RBP's (14,15).

PK-C and PK-C Assay -- Soluble PK-C was prepared from mouse brain (3) and PK-C-dependent phosphorylation of substrates [cRBP, cRABP, and histone III-S (Sigma Chemical Co.)] was determined as previously described (17) with slight modification as follows. Reaction mixtures contained 25 μg of substrate protein unless otherwise indicated, and 20 μM ATP as [γ-^{32}P]-ATP (20 Ci/mmol, Amersham-Searle Co., was diluted with unlabeled ATP, Sigma Chemical Co., to yield a final specific activity of 60 mCi/mmol), and reaction mixtures were incubated at 30°C for 5 min. Protein concentrations were determined by the Coomassie blue-binding assay using lysozyme as a protein standard (18).

Sodium dodecyl sulfate-polyacrylamide gel (SDS-PAG) electrophoresis and autoradiography were carried out according to the method of Hames and Rickwood (19).

RESULTS

Our experiments focused on determining if apo-cRBP and apo-cRABP could act as substrates for PK-C. Since both apo-RBP's contain approximately five serine, two tyrosine, and six threonine residues and share some homology with histone H-I, the predominant histone species in the histone III-S fraction, we felt that the PK-C assay parameters previously established with histone III-S provided an excellent system for evaluating the phosphorylation of cRBP and cRABP. Under such conditions (Table I), both apo-cRBP and apo-cRABP are capable of being phosphorylated by endogenous soluble protein kinases. Moreover, it is apparent that the phosphorylation of the RBP's as well as the histone III-S fraction is mediated predominantly by PK-C as evidenced by Ca^{+2} and phosphatidylserine (PS) sensitivity of the reaction; residual phosphorylation is minimally apparent in the purified apo-cRBP and apo-cRABP fractions and in the brain supernatant fraction. In a separate experiment, the apparent K_m for apo-cRBP was determined to be 6.2 μM, and 5.1 μM for apo-

TABLE 1
EFFECT OF VARIOUS COFACTORS ON THE PHOSPHORYLATION OF cRBP, cRABP, AND HISTONE III-S BY PROTEIN KINASE-C FROM 35K x g MOUSE BRAIN SUPERNATANT FRACTION*

	SUBSTRATE		
ASSAY MEDIUM	cRBP	cRABP	HISTONE III-S
Complete	5987	3508	3544
- Ca^{+2}	2737	2521	1936
- PS	879	658	652
- Ca^{+2}, - PS, - Mg^{+2}	247	356	--
- cRBP	202	--	--
- cRABP	--	247	--
- Histone III-S	--	--	157
- Supernatant	176	112	--

* Values are expressed as pmol P bound/min/mg soluble fraction protein and are the means of four determinations.

cRABP while the K_m value for the histone III-S fraction was estimated to be 10.8 μM. The apparent K_m for ATP was determined to be 12.4 μM in the presence of apo-cRBP and 2.6 μM in the presence of apo-cRABP. Further, both apo-cRBP and apo-cRABP were at least as effective as the histone III-S fraction substrate with apo-cRBP being ~1.7 times more effective on a μg basis than either cRABP or the histone III-S fraction.

Autoradiographic analysis (Figure 1-A) of the electrophoresed reaction mixtures confirms the PS sensitivity (lane 4, - PS) as well as the Mg^{+2} sensitivity (lane 7, - Mg^{+2}) of the phosphorylation of cRBP when compared with the complete mixture control (lane 6). The exclusion of Ca^{+2} from the reaction (lanes 3 and 5) reduced the lateral diffusion of the [^{32}P]-labeled cRBP in these lanes when contrasted with the respective complete mixture controls (lanes 2 and 6). This phenomenon appears to be due to the reduced salt concentration ratio between the lanes and the lateral gel and also explains the bilobular appearance of the radiolabeled bands. Furthermore, complexing all of the Ca^{2+} with an excess of EGTA precludes all phosphorylation at cRBP (lane 1). Phosphorylation of cRBP was also concentration dependent as indicated by increasing grain density for 10, 20, and 80 μg additions of apo-cRBP (lanes 2, 6, and 9, respectively). Interestingly, the addition of holo-cRBP (lane 8) reduced the phosphorylation of itself and the supernatant proteins. In contrast to the effect that the omission of incubation components such as PS, Ca^{+2} and Mg^{+2} had on [^{32}P]-incorporation into the cRBP band (Figure 1-A), these components or their omission had no effect on the electrophoretic banding pattern of the proteins in the assay mixtures (Figure 2-B). This latter observation suggests that changes in the assay mixture did not significantly alter protein migration but rather had a direct effect on PK-C activity.

Using the same reaction conditions, identical autoradiographic results were achieved for the apo-cRABP substrate (Figure 2-A). Again, PS sensitivity (lane 4, - PS) as well as Mg^{+2} sensitivity (lane 7, - Mg^{+2}) of the phosphorylation of apo-cRABP is indicated when compared to the respective

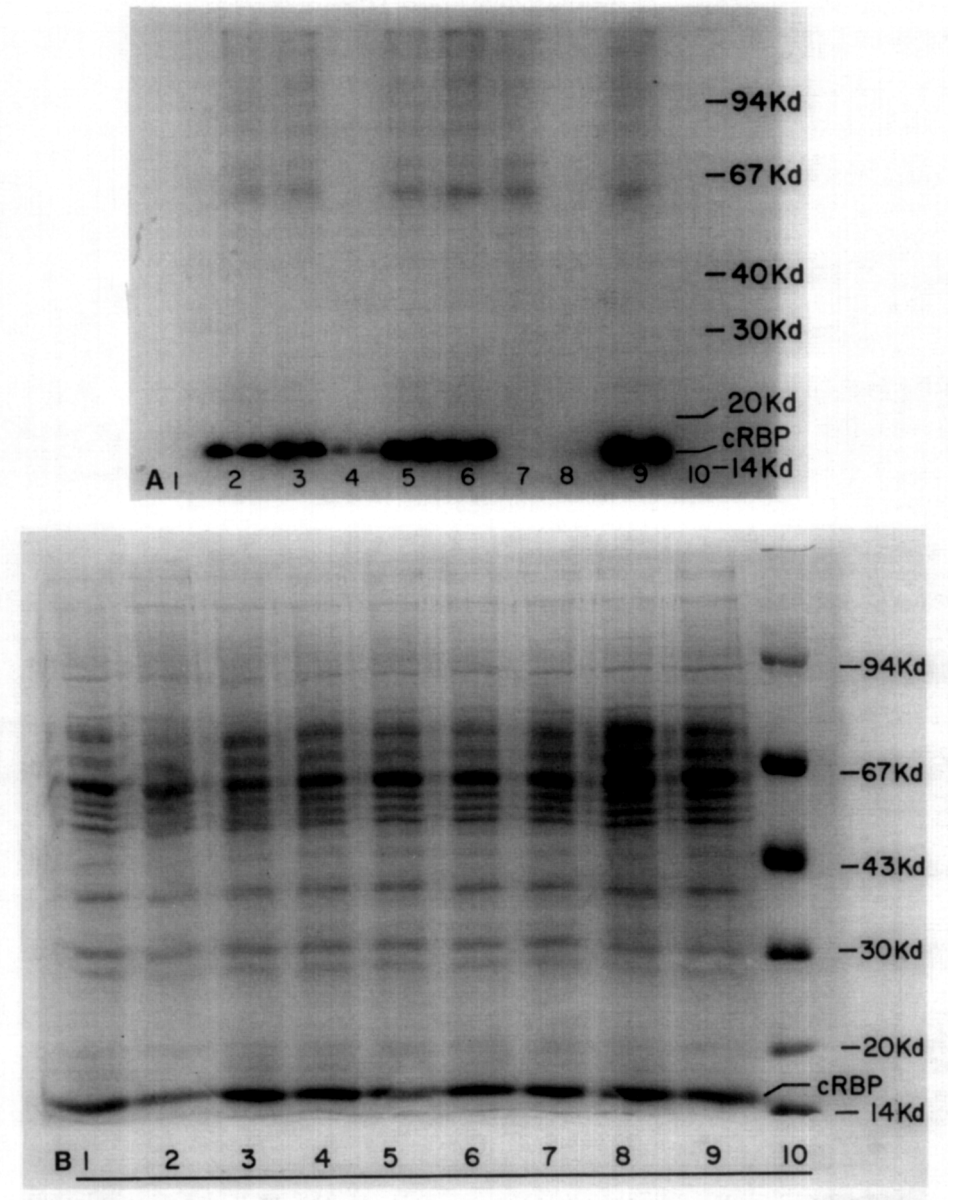

Figure 1 - (A) Autoradiographic Analysis of the PK-C Reaction Mixture Containing Purified Cellular Retinol-Binding Protein (cRBP) and (B) Corresponding Coomassie Blue-Binding Electrophoresis Pattern. The complete assay medium had the indicated components added or deleted: Lane 1, + 25 mM EGTA; lane 2, + 10 μg apo-cRBP; lane 3, + 10 μg apo-cRBP, - Ca^{+2}; lane 4, + 20 μg apo-cRBP, - PS; lane 5, + 20 apo-cRBP, - Ca^{+2}; lane 6, + 20 μg apo-cRBP; lane 7, + 20 μg apo-cRBP, - Mg^{+2}; lane 8, + 20 μg holo-cRBP; lane 9, + 80 μg apo-cRBP; lane 10, M_r markers, myoglobin, 14.0 Kd; trypsin inhibitor, 20.1 Kd; carbonic anhydrase, 30.0 Kd; ovalbumin, 43.0 Kd; bovine serum albumin, 67.0 Kd; and phosphorylase b, 94.0 Kd. Reaction mixtures (125 μl) were incubated for 5 min at 30°C and terminated by the addition of 100 μl of electrophoresis sample buffer. 150 μl of the terminated reaction mixture was then heated to 95°C for 5 min and applied to a 1.5 mm, 12.5% PAG. Electrophoresis and Coomassie Blue R-250 staining were accomplished as described. The resulting gel was quick-dried on an automatic gel dryer and applied to Kodak X-Omat AR film for 15 hr; the exposed film was developed according to the manufacturer's specifications.

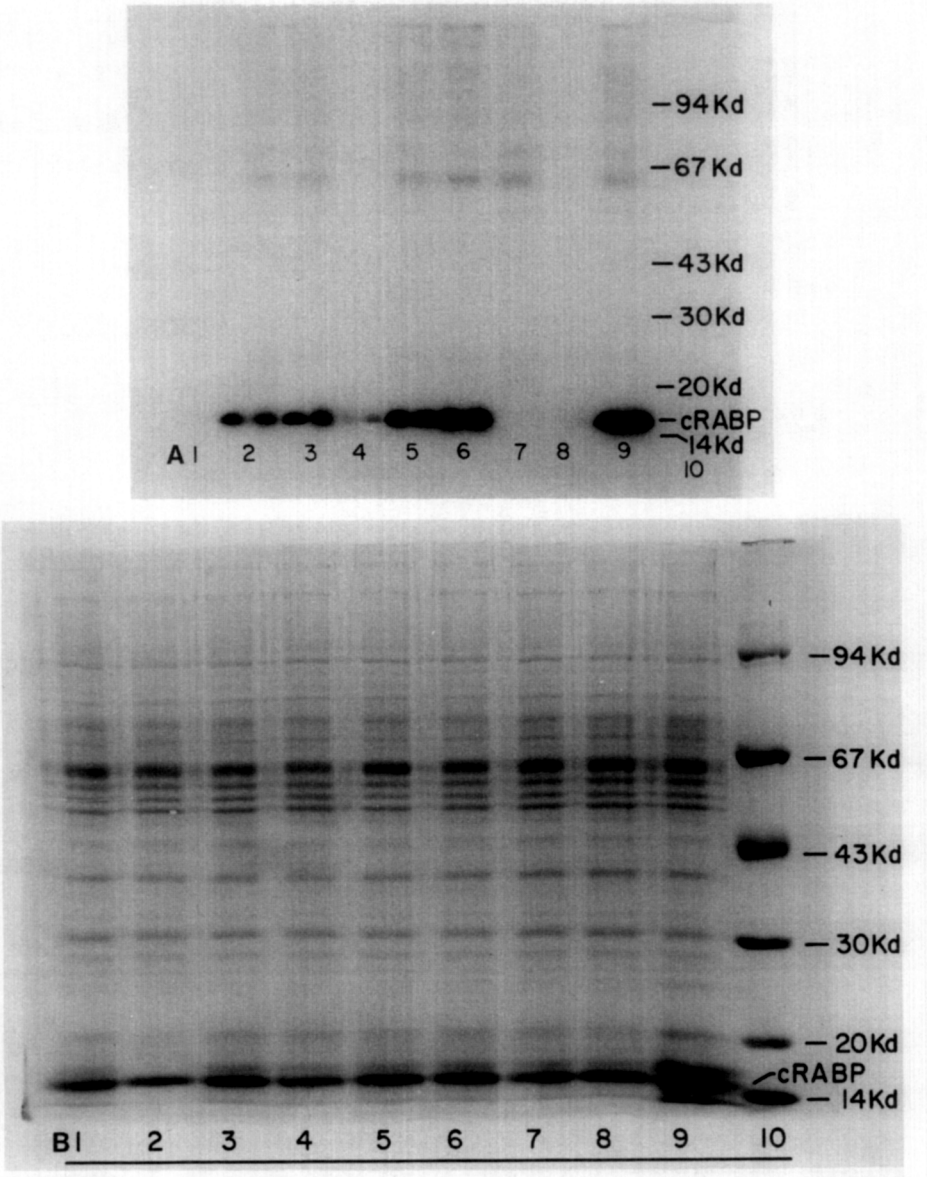

Figure 2 - (A) Autoradiographic Analysis of the PK-C Reaction Mixture Containing Purified Cellular Retinoic Acid-Binding Protein (cRABP) and (B) Corresponding Coomassie Blue-Binding Electrophoresis Pattern. Reaction mixtures as described had the indicated components added or deleted as in Figure 1 with cRABP-substituted for cRBP.

complete mixture control (lane 6). The elimination of Ca^{+2}, (lanes 3 and 5), produced a similar pattern in film grain density as that observed for cRBP when contrated with the respective complete mixture controls (lanes 2 and 6). The concentration-dependent phosphorylation of cRABP (10, 20, and 80 µg cRABP; lanes 2, 6, and 9, respectively) was also observed. Holo-cRABP also

reduced PK-C-dependent phosphorylation of itself and the supernatant proteins (lane 8). As in Figure 1-B, the addition or omission of assay components had no effect on the assay mixture protein pattern in SDS-PAG electrophoresis (Figure 2-B). Finally, we did observe a 16.4 Kd protein in the cRABP preparation; this protein migrated immediately above the cRABP indicated in Figure 2-B, Lane 9. However, excision and scintillation counting of the 16.4 Kd band and the cRABP band indicated that the [^{32}P]-activity resides with the cRABP as was the case for all other lanes in this experiment.

DISCUSSION

Retinoids are involved in the maintenance of normal differentiation of epithelia and overall organismic growth, and have been shown to be effective antineoplastic agents in numerous experimental systems (6,20,21). We have demonstrated in these experiments that both cRBP and cRABP, the apparent intracellular carriers of the retinoids, retinol and retinoic acid, respectively, are good substrates for PK-C, providing evidence for the possible interaction of RBP's and PK-C in vivo. Such interaction is also indirectly supported by the observation that in murine Lewis lung tumor cells, cryptic cytosolic cRABP which is phosphorylated and incapable of binding its ligand, retinoic acid, has been found although no phosphorylated cRABP could be found in the cytosol of normal cells (22). Thus, in TPA-induced tumorigenesis, PK-C phosphorylation of RBP's and inhibition of their ligand binding may result in the depletion of intracellular retinoids through normal metabolic pathways and the inability of retinoid-dependent target cells to take up and/or utilize active retinoids, producing an obligatory function deficiency. Such phenomena may explain the loss of efficacy of retinoids in blocking tumorigenesis when applied subsequent to promoters on the mouse skin.

We also demonstrated that saturated or holo-cRBP and holo-cRABP were capable of inhibiting PK-C activity as was evidenced not only by a reduction in their own phosphorylation but also by a lack of [^{32}P]-incorporation into brain cytosolic proteins (Figures 1A and 2A, lane 8); the latter observation

provides a second aspect to the interaction of TPA-induced tumorigenesis and retinoids. However, a third aspect also may be involved in which retinoids induce phosphatase enzyme activity and limit the efficacy of TPA-induced PK-C. In fact, retinoic acid has been shown to induce alkaline phosphatase activity in mouse uroendothelial cells (23), and our experiments do not rule out this possibility in the mouse brain system used here.

Finally, because cRBP and cRABP can act as protein kinase substrates, their phosphorylation may play a central role in tumorigenesis of retinoid-dependent cells and in controlling normal physiological aspects of retinoid action.

ACKNOWLEDGEMENTS: The authors would like to sincerely thank Dr. Curtis Ashendel, Purdue University, for his valuable discussions and Ms. Karen Denk for typing this manuscript. This research was supported by NIH Grants CA-07175, CA-22484, and CA-09020.

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