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ABSENCE OF PHOSPHORYLATION OF RETINOID-BINDING PROTEINS BY PROTEIN KINASE C *IN VITRO*

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Cellular retinol-binding protein, cellular retinoic acid-binding protein, and fetal cellular retinol-binding protein were purified to homogeneity and each polypeptide had a molecular weight of 16,000. Their apoproteins were not phosphorylated under the same conditions. Their holoproteins did not inhibit the phosphorylation of histone III-S by protein kinase C. Each of these observations is contrary to the results reported by Cope *et al.* (Biochem. Biophys. Res. Commun., 120, 593-601, 1984).

Receptors of epidermal growth factor (1,2), insulin (3), somatomedin (3), and transferrin (4,5) as well as certain contractile, cytoskeletal, membrane, and nuclear proteins (6) are phosphorylated by Ca^{++}-activated, phospholipid dependent protein kinase C. This kinase, which is activated by diacylglycerol, is a receptor for the 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-type tumor promoters, and the promoters, TPA, teleocidin and debromoaplysiatoxin, also activated the kinase (7,8). Recently Cope *et al.* reported that soluble protein kinase C isolated from mouse brain phosphorylates the apoproteins of cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP) *in vitro* and that the activity of protein kinase C was inhibited by these holoproteins (9).

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Abbreviations used: TPA, 12-O-tetradecanoylphorbol-13-acetate; CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid-binding protein; FCRBP, fetal cellular retinol-binding protein; SDS, sodium dodecylsulfate.

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Because these observations could provide new insight into the mechanism of control of metabolic processes by retinoids as well as establish a role for retinoid-binding proteins as inhibitors of tumor promotion, we attempted to confirm and extend these results.

Although we confirmed that protein kinase C isolated from mouse brain readily phosphorylated histone III-S, apo-CRBP, apo-CRABP, and the apo-form of fetal CRBP (FCRBP) were not phosphorylated at all. Likewise, holo-CRBP was not phosphorylated by protein kinase C. The data to support these results are presented here.

Materials and Methods

Cellular Retinoid-Binding Proteins: CRBP was purified from calf liver, CRBP and CRABP were from bovine adrenal glands and FCRBP was from bovine fetal liver. Their isolations were achieved by the method described by Ong and Chytil previously (10, 11), with slight modifications. Briefly, acid precipitation, batch treatment with CM-cellulose, DEAE-cellulose column chromatography, gel filtration on Sephadex G-50 and hydroxylapatite column chromatography were utilized. On some occasions, high performance liquid chromatography on Mono Q was used as a final step (12). Retinoid-binding proteins which were saturated with either all-trans-retinol or all-trans-retinoic acid were monitored by fluorescence measurements. CRBP, CRABP and FCRBP were each purified as a single protein by SDS-polyacrylamide gel electrophoresis. Their apoproteins were prepared by exposure to ultraviolet light at 366 nm using UVGL-58 Lamp (UVP, INC, CA, USA) for 9 h for CRBP, 9 to 11 h for CRABP, and 18 h for FCRBP (13).
Protein kinase C: Protein kinase C was partially purified by DEAE-cellulose chromatography of the soluble fraction of mouse brain (14,15).
Enzyme assay: The reaction mixture (125 µl) contained 25 µg of apoprotein, holoprotein, or histone III-S, 1 µg of partially purified protein kinase C and 10 µg of phosphatidylserine. In addition, 20 mM Tris-HCl buffer pH 7.4, 20 µM (r-^{32}P)ATP (1.0-1.7 x 10^5cpm/nmol), 8 mM magnesium acetate, 50 mM 2-mercaptoethanol and 1 mM $CaCl_2$ were included as reported previously (9). Incubation was carried out for 5 min at 30°C. The remaining radioactivity on the nitrocellulose membrane filter was counted.
SDS-polyacrylamide gel electrophoresis: The molecular weight of the three retinoid-binding proteins was determined by 15% polyacrylamide slab gel containing SDS (16).

Results and Discussion

The CRBP which was prepared from calf liver or bovine adrenal glands, the CRABP from bovine adrenal glands, and FCRBP from bovine fetal liver were each purified to a single polypeptide with a molecular weight of 16,000 determined by SDS polyacrylamide gel electrophoresis. Their molecular weights corresponded to those previously reported for bovine retinoid-binding proteins (17, 18). In contrast, the CRBP and

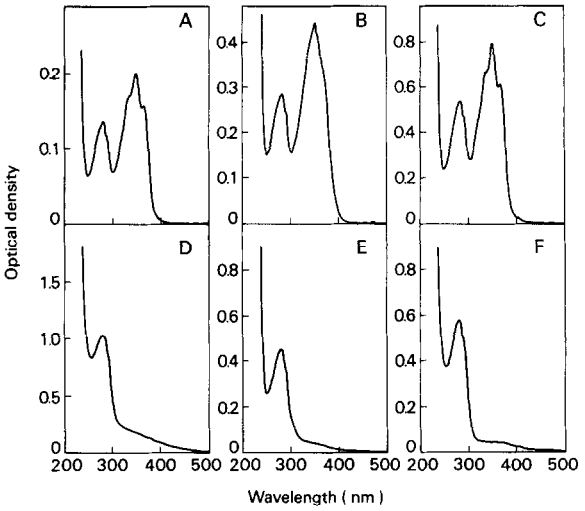

Fig. 1. Absorption spectra of CRBP(A), CRABP(B) and FCRBP(C). After exposure to ultraviolet light, spectra show apo-CRBP(D), apo-CRABP(E) and apo-FCRBP(F).

CRABP purified from calf liver and calf uterus, respectively, by Cope et al. were said to have a molecular weight of 14,600. It is not known if the difference between 16,000 and 14,600 is attributable to an error in determining molecular weight or if the proteins were truly different.

The absorption spectra of purified CRBP, CRABP, and FCRBP are shown in Fig. 1. Each spectrum shows two major peaks of absorption, the first (λmax, 280 nm) attributable to the binding protein and the second (λmax, 350 nm) to the presence of protein-bound retinol or all-trans-retinoic acid. The absorption spectra and fluorescence spectra of CRBP and CRABP were identical with those of retinoid-binding proteins isolated from bovine retina reported by Saari (17) and from rat liver and testis reported by Ong and Chytil (10,11). FCRBP isolated from bovine fetal liver showed the same absorption spectrum as CRBP. However, the fluorescence spectrum of FCRBP and CRBP differed at 350 nm and 370 nm and so they are distinctly different.

To prepare apoproteins, the three holoproteins were exposed to ultraviolet light for 9 h for CRBP, 9 to 11 h for CRABP, and 18 h for FCRBP. The absorption spectra of the treated holoproteins retained the first major peak at 280 nm but the peak at 350 nm was lost (Fig. 1).

Therefore, exposure to ultraviolet light was sufficient to produce the apoproteins. Using the same assay conditions as Cope et al., we examined the phosphorylation of histone III-S by protein kinase C isolated from mouse brain. After a 5 min incubation with the enzyme, 4.9×10^4 cpm of ^{32}P were incorporated into 25 ug of the histone. Thus histone III-S, commonly used as a substrate for protein kinase C together with phosphatidylserine, was readily phosphorylated under our experimental conditions. In contrast, apo-CRBP, apo-CRABP, and apo-FCRBP were not phosphorylated under the same conditions. Bovine serum albumin was used as a negative control; it was not phosphorylated. In addition, holo-CRBP could not serve as a substrate. Furthermore, in contrast to the report by Cope et al.(9), holo-CRBP, holo-CRABP, and holo-FCRBP did not inhibit the phosphorylation of hisone III-S by protein kinase C.

It is important to ascertain the reason for the fact that the earlier observations (9) were not repeatable. Although certain experimental details differed, these may not have been critical. For example, Cope et al. isolated protein kinase C from the 35,000 x g soluble supernatant fraction of mouse brain. In our experiments, the enzyme was purified by DEAE cellulose chromatography of a 100,000 x g supernatant fraction from mouse brain.

Treatment with ultraviolet light was used for preparing the apoproteins. The absorption spectrum of the binding proteins was tested every hour to determine when absorption at 350 nm disappeared. The duration of exposure that was required for destruction of the bound retinoid varied from 9 to 18 h depending on the protein. The effect of this treatment on the physical structure of the proteins is not known.

Perhaps the major reason for the inability to repeat the observations reported by Cope et al. resides in the retinoid-binding proteins themselves. Although the proteins that were prepared in each laboratory appeared capable of binding retinoids, they may not have been identical with respect to availability of amino acids that are susceptible to phosphorylation by protein kinase C. There are two possibilities.

First, the receptor proteins appear to have differed in molecular weight; the protein preparation used by Cope et al. (9) may have been partially degraded, exposing phosphorylatable amino acids. Second, ultraviolet light, used to inactivate the bound retinoids, causes varying degrees of change in proteins. Because the conditions used to inactivate the retinoid differed in the two laboratories, susceptibility to phosphorylation could differ.

In conclusion, the observations indicative of a mechanism for a vital control function for retinoids (9) requires confirmation before it can be recognized as being valid.

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References

1. Hunter, T., Ling, N., and Cooper, J.A. (1984) Nature 311, 480-483.
2. Iwashita, S., and Fox, C.F. (1984) J. Biol. Chem. 259, 2559-2567.
3. Jacobs, S., Sahyoun, N.E., Saltiel, A.R., and Cuatrecasas, P. (1983) Proc. Natl. Acad. Sci. USA. 80, 6211-6213.
4. May, W. S., Jacobs, S., and Cuatrecasas, P. (1984) Proc. Natl. Acad. Sci. USA. 81, 2016-2020.
5. Klausner, R.D., Harford, J., and van Renswoude, J. (1984) Proc. Natl. Acad. Sci. USA. 81, 3005-3009.
6. Kikkawa, U., and Nishizuka, Y. (1985) The Enzymes (E.G. Krebs and P.D. Boyer eds.), in press, Academic Press, Orlando, Florida, and London.
7. Nishizuka, Y. (1984) Nature 308, 693-698.
8. Fujiki, H., Tanaka, Y., Miyake, R., Kikkawa, U., Nishizuka, Y., and Sugimura, T. (1984) Biochem. Biophys. Res. Commun. 120, 339-343.
9. Cope, F.O., Staller, J.M., Mahsem, R.A., and Boutwell, R.K. (1984) Biochem. Biophys. Res. Commun. 120, 593-601.
10. Ong, D.E., and Chytil, F. (1978) J. Biol. Chem. 253, 828-832.
11. Ong, D.E., and Chytil, F. (1978) J. Biol. Chem. 253, 4551-4554.
12. Bonelli, F.C., and De Luca, L.M. (1985) Anal. Biochem. 147, 251-257.
13. Ong, D.E., Tsai, C.H., and Chytil, F. (1976) J. Nutr. 106, 204-211.
14. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S, and Nishizuka, Y. (1982) J. Biol. Chem. 257, 13341-13348.
15. Parker, P.J., Stabel, S., and Waterfield, M.D. (1984) EMBO J. 3, 953-959.
16. Laemmli, U.K. (1970) Nature 227, 680-685.
17. Saari, J.C. (1982) Methods in Enzymology vol. 81 (L. Packer ed.), pp. 819-826. Academic Press, New York.
18. Sundelin, J., Das, S.R., Eriksson, U., Rask, L., and Peterson, P.A. (1985) J. Biol. Chem. 260, 6494-6499.