THE PRINCIPAL GLUCOCORTICOID BINDING MACROMOLECULE IN HEPATOMA CELLS IN CULTURE IS SIMILAR TO CORTICOSTEROID BINDER II OF RAT LIVER CYTOSOL*

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Received April 17,1973

SUMMARY

Reuber H-35 hepatoma (H4-II-E-C3) and HTC cells are known to retain differentiated corticosteroid induced functions in cell culture and to bind corticosteroids to macromolecules in cytosol which subsequently enter the cell nuclear fraction. Using both cell types we have demonstrated the major macromolecular fraction in cytosol to have properties (elution position from DEAE columns, pI, ^{3}H-dexamethasone binding), very similar to those of rat liver corticosteroid Binder II which may be the hormone receptor.

Hepatomas have provided important sources for establishment of stable lines of cell culture. These cultures retain certain of the phenotypic functions which characterize a hormonal response in normal liver cells. The induction of tyrosine aminotransferase by corticosteroids is an example. Since we have already described the corticosteroid binding proteins of rat liver (1-4) and at least one of these proteins appears to be implicated in corticosteroid action (5), it is important to learn whether hepatomas retaining inducibility for tyrosine aminotransferase by corticosteroids also contain one or more of the components of the corticosteroid binding system typical of liver. In addition, since many liver type glucocorticoid responses have been deleted from hepatoma cells and the metabolism of steroids

*Supported by research grants AM-13535 and AM-08350 from the National Institute of Arthritis, Metabolic and Digestive Diseases and by CA-12227 to the Fels Research Institute.

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is greatly reduced, these cultures may show deletions of corticosteroid binding macromolecules which are not directly part of the "receptor" function involved in the induction of tyrosine aminotransferase, thus simplifying the task of identifying which of the binders constitutes the "receptor". In this connection, the corticosteroid binding protein of HTC cells, another hepatoma derived system as well as the Reuber hepatoma, has properties suggestive of its involvement in hormone action (6-8).

This paper describes in vitro cortisol binding in Reuber hepatoma-derived H4-II-E-C3 cells and in HTC cells. Evidence is presented showing that the major binding macromolecule in both hepatoma cell lines in culture is similar to corticosteroid Binder II in rat liver cytosol which is thought to be the main component of the hormone receptor in liver.

MATERIALS AND METHODS

Reuber hepatoma-derived H4-II-E-C3 cells grown in monolayer culture were stored frozen on dry ice. HTC cells were shipped frozen by B. Levinson and G.M. Tomkins. Cells were thawed gently at 4^{o} in 2 volumes of ice cold 0.05 M tris buffer, pH 7.5, containing 0.25 M sucrose. Cytosols were prepared as described previously (5). Nine ml of cytosol was diluted with 1 ml of 0.001 M tris (20% dimethylsulfoxide) containing 4×10^{-7} M ^{3}H-cortisol (New England Nuclear Corp., 44 Ci/mmole) for binding studies. The reaction mixture was then incubated at 4^{o} for 90 minutes. The extent of binding was similar in cytosols from cells stored frozen for up to 3 weeks.

Fractionation of cytosol proteins on columns of Sephadex G-25 followed by ion-exchange chromatography on DEAE-Sephadex A-50 columns has been described earlier (5). Partially purified cortisol binder from both cell types from DEAE-Sephadex A-50 chromatograms, was electrofocused (10) using LKB Instruments Inc. 110 ml columns and a pH 3 to 10 gradient. The assays for protein and radioactivity have been described elsewhere (5,11). The charcoal assay of Baxter and Tomkins (8) was used as an independent method to confirm that radioactivity was bound to protein in purified fractions.

The nature of the radioactivity bound to the major hepatoma cortisol binder was assessed by thin layer chromatography after exhaustive extraction of the protein-steroid complex with dichloromethane. The extraction 3 times with 2 volumes of dichloromethane resulted in 90% recovery of radioactive steroid. Thin layer chromatography was carried out using benzene: acetone (1:1 by volume) (5) and dichloromethane:acetone:benzene (7:2:1 by volume) (12) as described previously (5,10).

RESULTS AND DISCUSSION

When cytosols from hepatoma cells were incubated with 4.0×10^{-8} M ^{3}H-cortisol (as described in METHODS) 4 to 5% of the added cortisol radioactivity was associated with cytosol macromolecules. The binding was assessed after chromatography on columns of Sephadex G-25 (5,10) (Fig. 1.).

Further fractionation of the macromolecularly bound radioactivity on columns of DEAE-Sephadex A-50 resolved one major and some very minor steroid-protein complexes. This can be seen in Fig. 2A. The steroid-protein complexes eluting at 0.06 and 0.14 M KCl comprised about 95% and 4.0% respectively of the recovered bound radioactivity from cytosol. The remaining complexes eluting at 0 M and 0.23 M KCl each comprised only 1.0% of the cytosol radioactivity. Similar results were obtained with HTC cells (data not shown). These observations suggest that the hepatoma binding proteins are similar to those found in rat liver (Fig. 2B) although the relative amounts of radioactivity in the individual proteins are quite different so that the predominant macromolecule has properties similar to liver cytosol Binder II.

Since the hepatoma cell binding protein eluted at the same KCl concentration as rat liver Binder II it seemed possible it was either Binder II or a similar protein. To test this, a double label experiment was carried out. ^{3}H-Cortisol labeled bound radioactivity was prepared from HTC cytosol *in vitro* and a separate preparation of ^{14}C-cortisol labeled bound radioactivity was isolated from the liver cytosols of rats injected with ^{14}C-cortisol *in vivo*. As can be seen in Fig. 2C when the bound pools were mixed just before chroma-

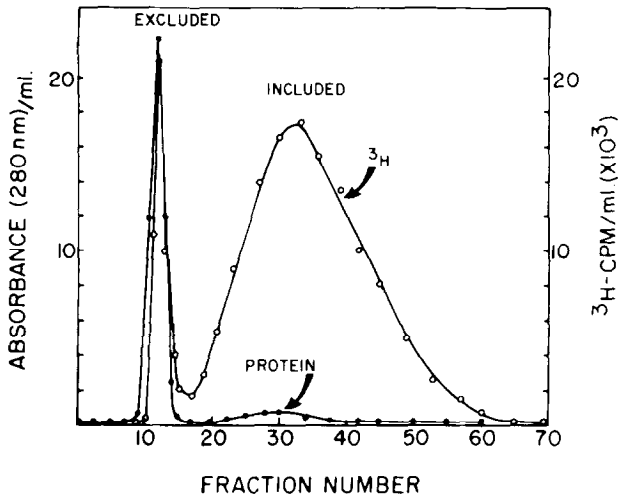

Fig. 1. Separation of bound and unbound radioactivity from HTC cytosol by Sephadex G-25 chromatography. Similar results were obtained with Reuber cell cytosol. Ten ml reaction mixtures containing cytosol and 4.8 x 10^{-8} M ^{3}H-cortisol were chromatographed on columns of Sephadex G-25 (2 x 50 cm) and eluted with 1 mM tris buffer. Four ml fractions were collected. The recovery of protein and radioactivity was 95%. The bound pools (excluded portions) (included portions) were concentrated and used for further purification.

tography and eluted from a column of DEAE-Sephadex A-50 the peak of bound ^{3}H from HTC eluted coincidently with the ^{14}C Binder II peak from rat liver. These data suggest that the hepatoma cell binding protein is similar to rat liver Binder II.

Isoelectric focusing of the HTC cortisol and dexamethasone binder from DEAE-Sephadex chromatography gives it a pH_I near 6.8 which is in good agreement with that observed with the rat liver cortisol Binder II in simultaneous experiments. Furthermore, electrofocusing of the double labeled peak from DEAE-Sephadex A-50 in a pH 3-10 gradient shows that radioactivity from cortisol Binder II and from the HTC or Reuber hepatoma binder is coincident and that the pH_I of both proteins is 6.8 suggesting that the two binding proteins are similar or identical. This experiment was repeated with the singly labeled major binder from Reuber hepatoma and its pI value was also 6.8 (Fig. 3). In both cases the peak fractions at pH 6.8 contained macromolecularly bound steroid by use of the charcoal binding assay (8). In spite of the appearance of the peak after isoelectrofocusing, the binder cannot be at all close

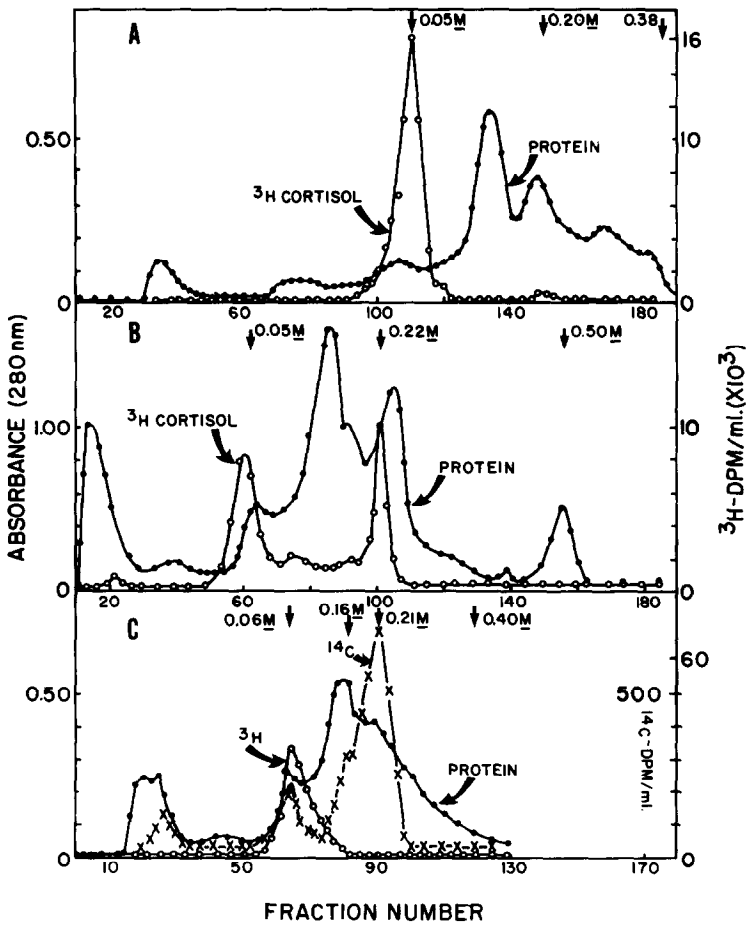

Fig. 2. DEAE-Sephadex chromatography of protein-bound radioactivity from rat liver and HTC cell cytosol. Similar results were obtained with Reuber hepatoma cell cytosol. The concentrated bound radioactivity from the cytosol of (A) HTC cells to which ^{3}H-cortisol was bound *in vitro* (B) rat liver to which ^{3}H-cortisol was bound *in vitro* (C) rat liver to which ^{14}C-cortisol was bound *in vivo* and HTC cell cytosol to which ^{3}H-cortisol was bound *in vitro*. The positions of the peaks of steroid-protein complexes were denoted by the KCl concentrations at which they eluted in the linear KCl gradient from 0 to 0.5 M. Fractions of 5.0 ml were collected. Recoveries of protein and radioactivity were 60 to 75%.

to homogeneity at this stage since we estimate that a purification in the range of 20,000 fold over the cytosol (in the case of rat liver) is required. However, it is likely that the purification factor required for the receptor from hepatoma cells may be substantially smaller. It is noteworthy that a similar situation was found with the estradiol receptor from calf uterus (13).

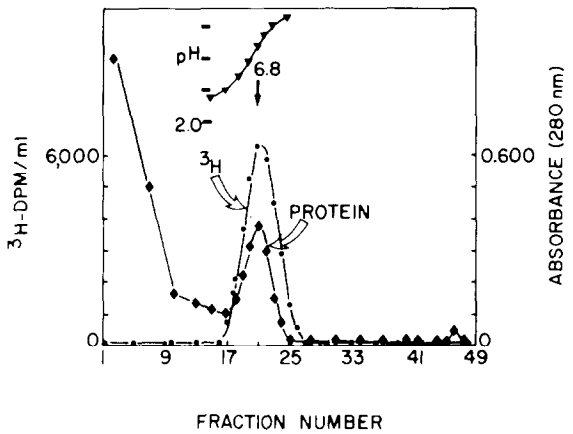

Fig. 3. Isoelectric focusing of the major cortisol binder from Reuber Hepatoma. Similar results are obtained with the binders from HTC cell cytosol. Electrofocusing of the concentrated pool from DEAE-Sephadex A-50 was carried out at 4° for 50 hours at 300 V and 0.5 ma. The pH_I of 6.8 of the peak of steroid complex is indicated in the chart. Recovery was quantitative.

Exhaustive extraction with dichloromethane (3,5) resulted in recovery of 90% of the radioactivity in the dichloromethane fraction. Thin layer chromatography in several systems showed that 90% of the radioactivity was unchanged cortisol and the remaining radioactivity consisted of equal amounts of tetrahydrocortisol and an unidentified steroid.

These data suggest that the Reuber and HTC hepatomas contain cortisol binding proteins similar to those found in rat liver, its parent tissue. Unlike the liver, cortisol Binder IV which contains transcortin is almost absent and the major binding protein in both cell types appears to be similar to corticosteroid Binder II which has been suggested to be the hormone receptor (4,14).

The receptor proteins from hepatoma cells and from liver cytosol have been implicated in hormone action, particularly in the induction of tyrosine transaminase (4,5,7,8,14). These proteins have many of the properties of receptor molecules (4,14). Hepatoma cell binder and liver cytosol binder are rapidly labeled with administered ^{3}H-cortisol, rat Binder II attaining maximal labeling within 5 minutes of intraperitoneal hormone administration

in vivo. They bind the unmetabolized hormone in vitro and in vivo and are saturated with hormone at physiologically inducing hormone concentrations. They also show binding specificity, associating maximally with glucocorticoids and anti-glucocorticoids. Finally, HTC receptor has been shown to mediate cortisol transfer to cell nuclei in cell free systems (15). Experiments with the rat liver system give similar results (unpublished experiments).

Additional unpublished studies with rat liver transplantable hepatomas in vivo show that varying amounts of "receptor" (Binder II) are present in the hepatomas studied and there is a direct correlation between the amount of Binder II and the degree of differentiation retained by the tumors (slow-growing compared to fast-growing). We hope that future studies will tell more about receptor. For example, is this protein adequate to facilitate complete induction of tyrosine transaminase in a fully inducible tissue (HTC cells have much lower basal tyrosine transaminase levels than liver, so the maximal induction levels are very different) or is the complete binding system found in rat liver (5) necessary? Alternatively, since corticosteroid Binder II is present in late fetal liver (5) and tyrosine aminotransferase is uninducible at this stage although inducing levels of hormone can be concentrated in the liver (5), the control of enzyme induction may reside at the level of the chromatin and may not depend on the quantity of hormone receptor available.

REFERENCES

1. Litwack, G., Sears, M.L. and Diamondstone, T.I., J. Biol. Chem., 238, 302 (1963).
2. Fiala, E.S. and Litwack, G., Biochim. Biophys. Acta, 124, 260 (1966).
3. Morey, K.S. and Litwack, G., Biochemistry, 8, 4813 (1969).
4. Litwack, G., Morey, K.S. and Ketterer, B. in B.R. Rabin and R.B. Freedman (Eds.), Effects of Drugs on Cellular Control Mechanisms, MacMillan, Ltd., London, 1971, p. 105-130.
5. Singer, S. and Litwack, G., Endocrinology, 88, 1448 (1971).
6. Gardner, R.S. and Tomkins, G.M., J. Biol. Chem., 244, 4761 (1969).
7. Baxter, J.D. and Tomkins, G.M., Proc. Nat'l. Acad. Sci. (USA), 65, 709 (1970).
8. Baxter, J.D. and Tomkins, G.M., Proc. Nat'l. Acad. Sci. (USA), 68, 932 (1971).

9. Pitot, H.C., Peraino, C., Morse, P.A., Jr., and Potter, V.R., Nat'l. Cancer Inst. Monogr., 13, 229 (1964).
10. Singer, S. and Litwack, G., Cancer Res., 31, 1364 (1971).
11. Weinstein, A., Medes, G. and Litwack, G., Anal. Biochem., 21, 86 (1967).
12. Randerath, K., Thin Layer Chromatography, Academic Press, New York and London, 1966, p. 129.
13. Puca, G., Nola, E., Sica, V. and Bresciani, F., in Methods of Enzymology, in press.
14. Rousseau, G.G., Baxter, J.D. and Tomkins, G.M., J. Mol. Biol., 67, 99 (1972).
15. Rousseau, G.G., Baxter, J.D., Benson, M.C., Garcea, R.L., Ito, J. and Tomkins, G.M., Proc. Nat'l. Acad. Sci., 69, 1892 (1972).