Vol. 143, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 27, 1987 Pages 98-103

IDENTIFICATION OF A CHARACTERISTIC CYTOSOLIC POLYPEPTIDE OF RAT PRENEOPLASTIC HEPATOCYTE NODULES AS PLACENTAL GLUTATHIONE S-TRANSFERASE

T.H. Rushmore, R.N.S. Sharma, M.W. Roomi, L. Harris, *K. Satoh, *K. Sato, R.K. Murray, and E. Farber

Departments of Pathology and of Biochemistry,
Medical Sciences Building, University of Toronto,
Toronto, Ontario, Canada M5S 1A8
*Department of Biochemistry,
Hirosaki University School of Medicine,
Hirosaki, Japan

Received December 31, 1986

Evidence is presented that a distinctive type of glutathione-S-transferase (GSHTase-P), and a cytosolic polypeptide of M_r 52,000 (P-52), each appearing in greatly increased amounts in hepatocyte nodules during liver carcinogenesis in the rat, are so far indistinguishable. The probable identity of the two polypeptides was established with the use of SDS polyacrylamide gel electrophoresis and Western blot techniques with purified GSHTase-P and P-52 and their respective antibodies and by comparison of the sequence of the first 26 N-terminal amino acids. Since the enzyme and the polypeptide are each considered to be the best available early markers for hepatocyte nodules, as putative precancerous lesions, their probable identity makes them attractive cellular components for in depth studies on their transcriptional and translational regulation and their use in new approaches to the sequential analysis of liver carcinogenesis.

Cytosol from preneoplastic hepatocyte nodules (HN), generated in six different models of liver carcinogenesis, showed a series of differences on SDS-PAGE from that of normal liver (1). A prominent difference was a marked increase in the amount of a polypeptide of M_r 26,000 (P-26), tentatively considered previously to have a molecular weight of about 21,000 (1). Recently, Sato and co-workers have isolated and purified a

Abbreviations: GSHTase-P, glutathione-S-transferase (EC2.5.1.18); HN, hepatocyte ("hyperplastic", "neoplastic") nodules; GluTase, γ-glutamyl-transferase (EC2.3.2.2); RH, resistant hepatocyte; PMSF, phenylmethyl-sulfonylfluoride; CM-sephadex, carboxymethyl cellulose-sephadex; GSH, glutathione; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl-sulfate-polyacrylamide gel electrophoresis; TBS, 20 mM Tris, 500 mM NaCl, pH 7.5; TTBS, TBS containing 0.05% Tween-20; PBS, 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.2.

0006-291X/87 $1.50

neutral form of GSHTase from rat placenta, GSHTase-P, and have shown it to be present in relatively high concentrations in HN, in comparison to normal control liver (2-5). Using the anti-GSHTase-P antibody, this enzyme was shown to be markedly increased and localized in GluTase-positive foci and in HN (5).

Since GSHTase-P and P-52 have similar molecular weights and appear in HN under similar biologic conditions, we postulated that they were closely related if not identical (4,6). Through the use of purified GSHTase-P and P-52 and their respective antibodies and of SDS-PAGE and Western blot techniques, evidence is presented for the close similarity if not identity of the two liver nodule polypeptides. This conclusion is supported by the observation that the sequences of the first 26 N-terminal amino acids are identical.

MATERIALS AND METHODS

Resistant hepatocyte (RH) nodules were produced by the method of Solt and Farber (7) in male Fischer 344 rats. Prior to harvesting the nodules, the livers were perfused with ice cold 0.25 M sucrose containing 1 mM PMSF (Sigma) as protease inhibitor and 1 mM EDTA. The nodules, grayish-white and up to 1 cm in diameter, were dissected free from the surrounding pale brownish red liver. Cytosol fractions from nodules and from control normal livers were prepared by homogenizing tissue (1 gm per 10 ml of medium) in 0.25 M sucrose containing PMSF and EDTA. Homogenates were centrifuged at 105,000 x g for 1 hr at 4°C and the clear supernatants were frozen at -70° until used. Protein estimaton was by the method of Lowry et al. (8), using bovine serum albumin as standard.

The GSHTase-P was purified from the cytosol of rat placenta at the 15th day of gestation (2), by sequential chromatography on CM-Sephadex C-50, hydroxylapatite and GSH affinity columns and finally on immunoaffinity columns coupled with antibodies against GSHTase AC subunits and GSHTase BL subunits to remove very small amounts of contaminating AC and BL GSHTases. Highly purified preparations of P-52 were prepared from the cytosol of HN by sequential chromatography on S-hexylglutathione, CM-Sephadex and DEAE-Sephacel columns. Antibodies to purified GSHTase-P and to purified P-52 were prepared in rabbits by intracutaneous injections with complete Freund's adjuvant.

SDS-PAGE was performed as described previously (1). Polypeptides from unstained SDS-PAGE gels were electrophoretically transferred to nitrocellulose paper with a Trans-blot apparatus (Bio-Rad) at 85 volts for 4 hours at 4°C using the buffer system of Towbin et al. (9). After transfer, the P-26 or GSHTase-P subunit was visualized by immunoblot analysis using either antibody to P-52 or GSHTase-P (10,11).

RESULTS AND DISCUSSION

Fig. 1A shows a typical polypeptide pattern on SDS-PAGE of cytosols from control rat liver and rat liver HN and of purified P-26 and GSHTase-P. The band appearing at about 26,000 molecular weight region in the HN cytosol and the correspondence between the position of this band and those of P-52 and GSHTase-P are evident. The immunoblot (Western blot) corresponding to the SDS-PAGE and after reacting with the antibody to GSHTase-P is shown in Fig. 1B. The similarity in the properties of P-52 and GSHTase-P in respect to their reaction with antibodies to GSHTase-P is readily apparent.

In Fig. 2 is shown a similar set of data, with the use of antibodies prepared against P-52 rather than against GSHTase-P. With the use of this antibody, the small amount of P-52 that is present in the cytosol from control liver (1) is now visible in the immunoblot preparation (B). The reaction of this antibody preparation with GSHTase-P is clearly evident. Thus, the GSHTase-P and P-52 are in similar positions in SDS-PAGE and antibodies prepared against each purified preparation react with both preparations. Also, in histological sections of control livers and livers with HN, the patterns of staining using the two different antibody preparations are identical (5, Cameron and Rushmore, personal communication).

A similar tentative conclusion in respect to GSHTase-P, P-52 and nodules has been suggested by Jensson et al. (12). These authors designate GSHTase-P as GSHTase 7-7, a GSHTase which is present at a very low level in rat liver but at relatively high levels in kidney and lung. In their study, purified P-52 from HN was not used. A glutathione-S-transferase, a homodimer resembling GSHTase-P and GSHTase 7-7, has been purified from rat primary hepatomas (13). This GSHTase, like GSHTase 7-7 and unlike several other GSHTases, has high activity towards ethacrynic acid (14). Highly purified P-52, like GSHTase 7-7, also has high activity toward ethacrynic acid.

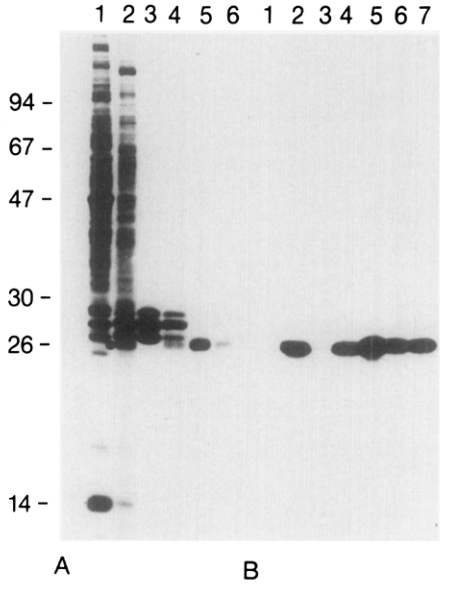

Fig. 1. Analysis of cytosolic polypeptides of control livers and of hepatocyte nodules.

A. SDS-PAGE (12.5% polyacrylamide) using 20 µg protein/lane except for lane 6 for which about 5 µg protein were used. The gel was stained with Coomassie Blue B. The markers for estimation of M_r were phosphorylase B (94,000), bovine serum albumin (67,000), ovalbumin (47,000), carbonic anhydrase (30,000) and lysozyme (14,000).

1, control liver; 2, HN; 3, affinity purified cytosol (step 1) from control liver; 4, affinity purified cytosol (step 1) from HN; 5, highly purified P-52; 6, purified GSHTase-P.

B. Immunoblot (Western blot) analysis of a gel identical to that shown in A using GSHTase-P antibody. After transfer, the nitrocellulose paper was treated with Blotto (5% non-fat dry milk in PBS) for 1 hour at room temperature and then incubated in Blotto containing antibody to GSHTase-P (diluted 1:200) for 4 hours at room temperature (18). The paper was then washed in Blotto and treated with ^{125}I Protein A for 2.5 hours at room temperature (0.5 µCi/10 ml). The blot was washed in PBS and the polypeptides reacting with the primary antibody were detected by autoradiography. Lanes 1,2,3,4,5 and 7 correspond to lanes 1-6 in Fig. 1A. In lane 6, 5 µg of purified P-52 was used. The antibody used was against GSHTase-P.

Preliminary data on the sequence of amino acids in P-52 indicate that the first 26 N-terminal amino acids is identical to that found in GSHTase-P (15) and in GSHTase 7-7 (14). Thus, several lines of evidence indicate the almost certainty that P-52, GSHTase-P and GSHTase 7-7 are the same protein with comparable enzyme activities.

Since HN have a characteristic biochemical phenotype that confers unusual resistance of this new liver cell population to many xenobiotics and

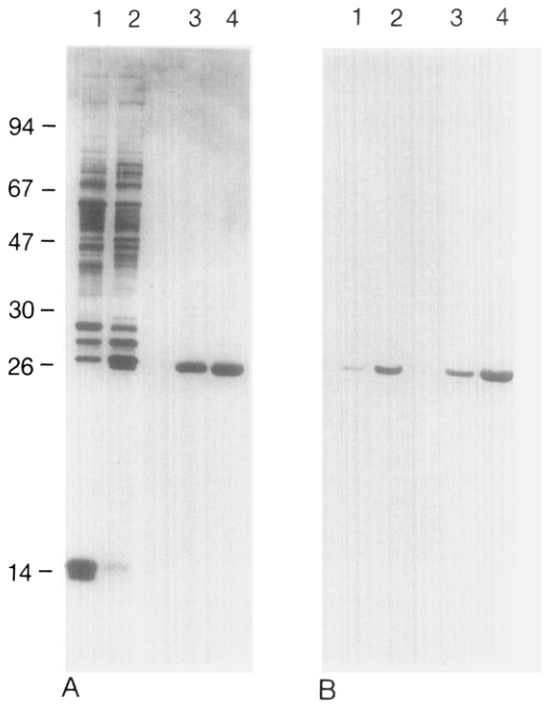

Fig. 2. Analysis of cytosolic polypeptides of control livers and of hepatocyte nodules.

A. SDS-PAGE (15% polyacrylamide) using 6 µg protein/lane. The gels were stained with silver nitrate as described by Wray et al. (19). The markers for estimation of M_r were the same as in Fig. 1A. 1, control liver; 2, HN; 3, P-52, 4, GSHTase-P.

B. Immunoblot (Western blot) analysis of a gel identical to that shown in Fig. 2A. After transfer, the nitrocellulose paper was treated with 3% gelatine in TBS for 1 hour at room temperature, then incubated in TTBS (TBS containing 0.05% Tween-20) containing antibody to P-52 (diluted 1:300) for 4 hours at room temperature. The paper was washed in TTBS and incubated in TTBS containing 1% gelatin ml goat anti-rabbit HRP-conjugated IgG (Bio-Rad) diluted 1:3000 for 1 hour at room temperature. The polypeptides reacting with the primary antibody were visualized by immersion in development solution (100 ml TBS containing 60 µl 30% H_2O_2 and 60 mg 4-chloro-1-Napthol) for 10 min at room temperature. The lanes are the same as in Fig. 2A.

to exposure to toxic environmental components (16,17), it is attractive to consider GSHTase-P as another component of this resistance spectrum. Since this polypeptide shows a large increase in activity and in amount in the putative preneoplastic and precancerous hepatocytes, it might well prove to be a useful focus for studies of altered gene expression during carcinogenesis and in the development of a novel assay for initiation and promotion of hepatocarcinogenesis.

ACKNOWLEDGEMENTS

This work was supported by grants from NCI and MRC of Canada and the NCI (NIH). THR is in receipt of an NIH Fellowship.

REFERENCES

1. Eriksson, L.C., Sharma, R.N., Roomi, M.W., Ho, R.K., Farber, E. and Murray, R.K. (1983). Biochem. Biophys. Res. Commun. 117, 740-745.
2. Sato, K., Kitahara, A., Satoh, K., Ishikawa, T., Tatematsu, M. and Ito, M. (1984). Gann 75, 199-202.
3. Kitahara, A., Satoh, K., Nishimura, K., Ishikawa, T., Ruike, K., Sato, K., Tsuda, H. and Ito, N. (1984). Cancer Res. 44, 2698-2703.
4. Satoh, K., Kitahara, A., Inaba, Y., Hatayama, I. and Sato, K. (1985). Proc. Natl. Acad. Sci. USA 82, 3964-3968.
5. Tatematsu, M., Mera, Y., Ito, N., Satoh, K. and Sato, K. (1985). Carcinogenesis 6, 1621-1626.
6. Farber, E. (1984). Cancer Res. 44, 5463-5474.
7. Solt, D.B. and Farber, E. (1976). Nature 263, 701-703.
8. Lowry, O.H., Rosebrough, K.J., Farr, A.L. and Randall, O. (1951). J. Biol. Chem. 193, 265-275.
9. Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350-4354.
10. Crawford, L.V. and Lane, D.P. (1977) Biochem. Biophys. Res. Comm. 74, 323-329.
11. Brockmann, W.W. (1978). J. Virology 25, 860-870.
12. Jensson, H., Eriksson, L.C. and Mannervik, B. (1985). Febs. Letters 187, 115-120.
13. Meyer, D.J., Beale, D., Tan, K.H., Coles, B. and Ketterer, B. (1985). Febs. Letters 184, 139-143.
14. Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M.K., Warholm, M. and Jörnvall, H. (1985). Proc. Natl. Acad. Sci. USA 82, 7202-7206.
15. Suguoka, Y., Kano, T., Okuda, A., Sakai, M., Kitagawa, T. and Muramatsu, M. (1985). Nucleic Acids Res. 13, 6049-6057.
16. Roomi, M.W., Ho, R.K., Sarma, D.S.R. and Farber, E. (1985). Cancer Res. 45, 564-571.
17. Farber, E. and Sarma, D.S.R. (1987). Lab. Invest. in press.
18. Johnson, D.A., Gautsch, J.W., Sportsman, J.R. and Elder, J.H. (1984). Gene Anal. Technol. 1, 3-8.
19. Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. (1981). Anal. Biochem. 118, 197-203.