Volume 308, number 2, 207–210 FEBS 11427 August 1992

Rat liver BiP/GRP78 is down-regulated by a peroxisome-proliferator, clofibrate

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Received 29 May 1992

Administration of clofibrate in rat results in down-regulation of several liver proteins and a vast induction of peroxisomal proteins. One protein was identified as BiP/GRP78 using antibodies and cDNA cloning. The level of mRNA was reduced by the drug.

1. INTRODUCTION

Peroxisomes are found in almost every eukaryotic cell [1] and carry out several metabolic functions [2]. Recent studies on peroxisomes have revealed remarkable information about the targeting signal [3], transcriptional activation of peroxisomal genes [4,5], peroxisomal assembly factors [6,7], and the cause of peroxisome-deficient Zellweger syndrome [8]. Another interesting aspect of peroxisomes is that the organelles proliferate remarkably well in rodent hepatic parenchymal cells with the administration of structurally diverse xenobiotic agents, peroxisome proliferators [9]. These agents also cause hepatomegaly and some of them are non-genotoxic but have a hepatocarcinogenic property [10]. Most studies on peroxisome proliferators have been focused on characterization of the specific induction of peroxisomal enzymes and their effects on cellular functions. Hepatomegaly includes activation of cell division but it has not been clarified whether the activation is dependent on or independent of the proliferation of peroxisomes.

We have been interested in the possibility that peroxisome proliferators may affect not only peroxisome-related cellular events but also other basic and/or liver-specific cellular functions unrelated to peroxisomes. In this study, we describe the down-regulation of an endoplasmic reticulum (ER) protein, BiP/GRP78 by a peroxisome proliferator, clofibrate. Reduction of the protein can have diverse effects on the structures and functions of the proteins that pass through the ER with the aid of BiP/GRP78.

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2. MATERIALS AND METHODS

2.1. *Materials*

Clofibrate (ethyl 2-(*p*-chlorophenoxy)isobutyrate) was purchased from Sigma (St. Louis, USA). Restriction enzymes, DNA polymerase I, Klenow fragment of DNA polymerase I, and T4 DNA ligase were obtained from Toyobo (Osaka, Japan). Normal and reverse sequence primers were from Takara Shuzo (Kyoto, Japan). [α-^{32}P]dCTP(3000 Ci/mmol) and affinity purified ^{125}I-protein A were purchased from ICN (Costa Mesa, USA) and DuPont/NEN (Wilmington, USA), respectively.

2.2. *Animals and subcellular fractionation*

Male Fischer F-344 rats were fed a control diet (CE7, Clea Japan) or a diet containing 0.5% clofibrate. After 2 weeks, rats were anesthetized with ether and decapitated. Postnuclear fractions were prepared from the livers as described [11]. The plasma membranes from the rat livers were obtained by the method of Hubbard et al. [12].

2.3. *Immunoblot analysis*

Protein samples were run through SDS polyacrylamide gel and transferred to nitrocellulose filters. The sheets were incubated with antibodies and the antigen-antibody-[^{125}I]protein A complex was detected by autoradiography as described [13]. The anti-TMG (tritosome membrane ghost) sera were obtained by injecting TMG [14] (a gift from Dr. Y. Moriyama) into a rabbit.

2.4. *cDNA cloning and identification of the positive clones*

A rat liver cDNA library in lambda gt11 was screened by anti-TMG antibodies according to [15]. The antibodies specific to the fusion protein expressed by the positive phage were purified and used in immunoblot analysis for further characterization. The nucleotide sequence of the cDNA insert was determined as previously described [16]. Homology searches were performed using the DNASIS program (Hitachi Software Engineering, Yokohama, Japan) and data bases, GenBank R66.0 and EMBL R25.0.

2.5. *Northern blot analysis*

Free and membrane-bound polysomes were purified from the rat livers according to [17]. Total polysomal RNAs (10 μg) were electrophoresed on 1.5% agarose/formamide gels, transferred to nitrocellulose filters, and hybridized with cDNA probes by the standard method [18]. cDNA clones used were isolated in our laboratory ([19] and K. Motojima et al., in preparation).

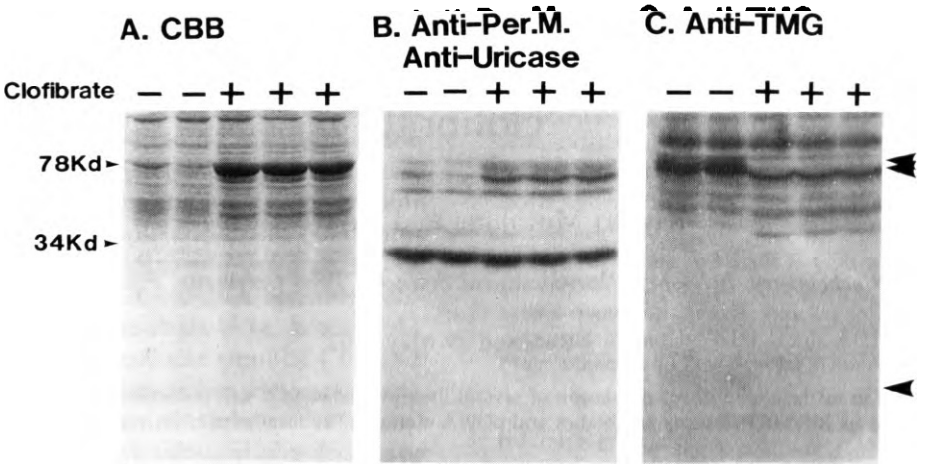

Fig. 1. Immunoblot analysis of postnuclear proteins from control and clofibrate-fed rats. A 50 μg protein from individual control (–) or clofibrate-fed (+) rats was separated on a SDS-polyacrylamide gel in duplicate. After electroblotting, the gel was stained with Coomassie blue (A) and the blotted filters were probed with the mixture of anti-peroxisomal membrane and anti-uricase antibodies (B) or with anti-TMG antibodies. Arrowheads on the right indicate the positions of the proteins whose levels were reduced by administration of clofibrate.

3. RESULTS

3.1. *Detection of the proteins down-regulated by clofibrate*

To detect proteins in the rat liver with levels that are decreased by feeding a diet containing clofibrate, we examined several antibodies by immunoblotting, using the protein samples of postnuclear supernatant fractions from control and clofibrate-fed rats. Neither Coomassie blue staining of the one-dimensional gels nor immunoblotting using several mono-specific antibodies recognized such proteins, but anti-TMG antibodies that recognize many lysosomal and some non-lysosomal proteins found three down-regulated proteins (Fig. 1); their molecular weights are 80 kDa, 78 kDa, and 24 kDa, respectively.

3.2. *Identification of one protein as BiP/GRP78*

To identify the proteins down-regulated by clofibrate, we first screened a rat liver expression cDNA library using the anti-TMG antibodies. The affinity-purified antibodies obtained from the total antibodies using the fusion protein from a positive phage were then checked for their reactivities against normal or clofibrate-fed rat liver proteins in the postnuclear fraction. Finally, the cDNAs were identified by nucleotide sequencing and homology searches in data bases.

By immunoscreening, we obtained 6 weakly and 4 strongly positive cDNA clones. The results of immunoblot analyses with affinity-purified antibodies using two of the four strong phages are shown in Fig. 2. The antigens they recognized have indistinguishable molecular weights of 78 kDa and the level of the protein is reduced by clofibrate. The molecular weight of the protein is similar to that of the peroxisome proliferation-associated protein, enoyl-coenzyme A hydratase-3-hydroxylacyl-coenzyme A dehydrogenase. Because of the vast induction of the latter protein (see Fig. 1A), the possibility of a blotting artifact or a masking effect was examined by mixing the protein samples followed by immunoblot analysis, but was excluded even though a weak masking effect was observed (data not shown).

For identification of the cDNAs, the nucleotide sequence of one cDNA was determined and homology searches were conducted in data bases. The cDNA was identified as one for rat BiP/GRP78, a member of the HSP70 family and a resident protein in the ER, as shown in Fig. 3. The cDNA corresponds to the published sequence from positions 1,116 to 1,800, and amino acid residue numbers from 354 to 582 [20]. Three out of four strongly positive clones were concluded to be those for BiP/GRP78 by cross hybridization. The other remains to be characterized.

3.3. *Northern blot analysis*

To confirm and clarify the step of down-regulation of BiP/GRP78 by clofibrate, levels of the mRNA in control and clofibrate-fed rat livers were analyzed by Northern blot hybridization. For RNA samples, free and membrane-bound polysomal RNAs were purified, and stringent, hybridization/washing conditions were employed to avoid cross hybridization with the mRNAs for the cytoplasmic HSP70 protein families.

As shown in Fig. 4, the BiP/GRP78 mRNA was detected on membrane-bound polysomes and its level was reduced to less than 50% of control in the liver of rats fed clofibrate for two weeks. The reduction was evident but the ratio was not as significant as that of the protein (See Figs. 1 and 2). This may be because the level of the protein in the liver of the clofibrate-fed rat was somewhat underestimated by the masking effect of a largely induced peroxisomal protein in the protein blotting pro-

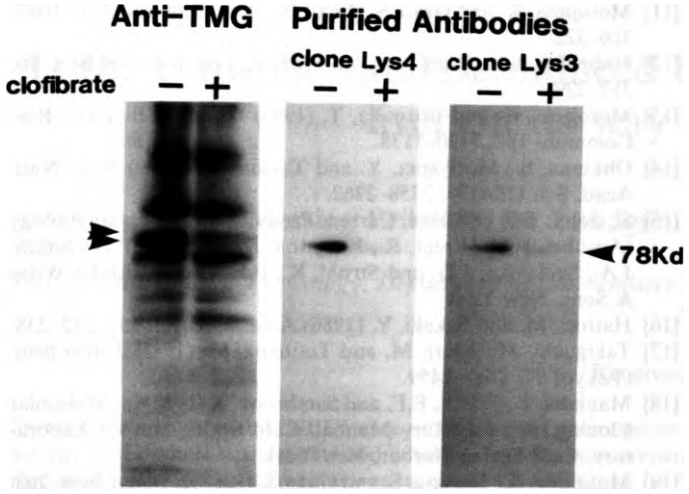

Fig. 2. Immunoblot analysis of the antigens recognized by the immunoaffinity-purified antibodies. A 50 μg postnuclear protein from control (−) or clofibrate-fed (+) rat was separated on a gel in triplicate. After electroblotting, one filter was probed with total anti-TMG antibodies and the remaining two were probed with antibodies affinity-purified from the total antibodies using the fusion proteins expressed in *E. coli* harboring clone Lys4 or clone Lys3.

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--- β gal      354 (Rat BiP/GRP78) 582
-----GluPheArgSerAsp--------LysGluLysProSTOP
-----gaattccgATCTGAT--------AAAGAGAAgcttgaattc---
     EcoRI  (1116----------1900)      EcoRI
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Fig. 3. Summary of the nucleotide sequence and the deduced amino acid sequence of clone Lys4 cDNA, in comparison with the published sequences of BiP/GRP78 [20].

cedure, and because an important step in regulation of the level of the protein is translational or post-translational.

4. DISCUSSION

BiP/GRP78 is an ER protein playing multiple roles in the process of ER translocation including folding, retention, and assembly of proteins in the ER by protein–protein interactions [21]. Reduction [22] and elevation [23] of the level of BiP/GRP78 in the ER have been shown to affect the efficiency of protein secretion. Recent studies have revealed that post-translational modifications of BiP/GRP78 by both phosphorylation and ADP-ribosylation [24], and its form, whether free or aggregated, are important in its function [25]. One-dimensional SDS-PAGE analysis in our present experiment did not distinguish the different forms of BiP/GRP78, but the reduction of the protein in hepatocytes can affect the efficiency of transport of many proteins that pass through ER with the aid of BiP/GRP78. Our preliminary experiment to compare the protein compositions of the purified plasma membranes [12] by SDS-PAGE suggested several differences between control and clofibrate-fed rat samples. Identification of the reduced proteins, however, must await further analysis using specific antibodies because the plasma membrane fractions, though enriched more than 20-fold over the starting homogenates, contained other membranes including those of proliferated peroxisomes.

Using specific antibodies, Bartles et al. [26] reported down-regulation of five plasma membrane proteins of rat liver by administration of a peroxisome proliferator. These include the epidermal growth factor receptor and the asialoglycoprotein receptor, which may be assembled in the ER with the aid of BiP/GRP78.

Reduction of several plasma membrane proteins including receptors presumably caused by down-regulation of BiP/GRP78 in the ER suggests the possibility that cellular signalling in hepatocytes of a peroxisome-proliferator-fed rat is perturbed from the normal state. Such perturbation may cause hepatomegaly and, finally, malignant transformation.

Though further detailed analyses are clearly necessary to establish causal relation among the proliferation of peroxisomes, down-regulation of BiP/GRP78, and hepatomegaly leading to malignant transformation, the

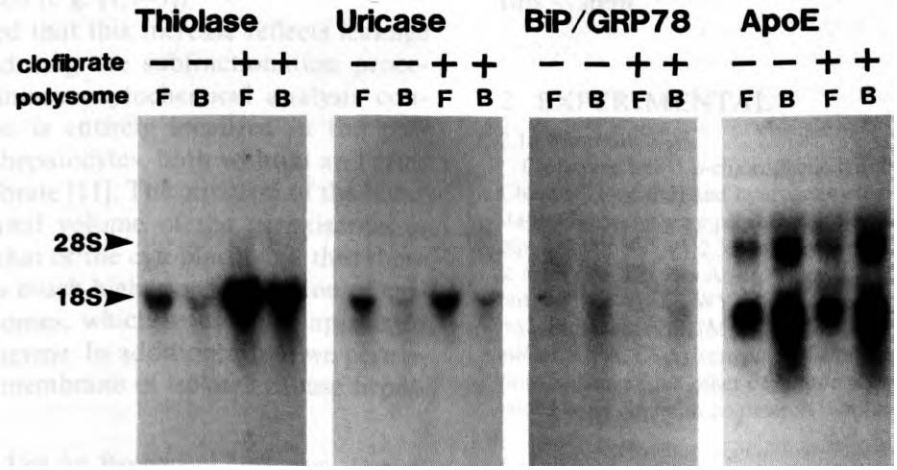

Fig. 4. Northern blot analysis of polysomal RNA from control and clofibrate-fed rats. A 10 μg sample of total RNA extracted from free (F) or membrane-bound (B) polysomes purified from the livers of control (−) or clofibrate-fed (+) rats was probed with thiolase, uricase, BiP/GRP78, or apoE cDNA. The positions of 28S and 18S ribosomal RNA are indicated on the left.

idea that peroxisome proliferators may cause hepatomegaly and hepatocarcinogenesis by perturbing fundamental cellular processes will be challenging to the oxidative stress hypothesis that emphasizes leakage of hydrogen peroxide from the proliferated peroxisomes and its damage to proteins and/or DNA [10].

Acknowledgments: We are grateful to Drs. T. Imanaka and Y. Moriyama for their generous gifts of the polysomes, anti-peroxisomal membrane antibodies and the TMG fractions.

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