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Negative correlation with liver cell division of a 38 kilodalton protein whose phosphorylation is enhanced by *ras* and G-proteins

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We showed earlier that the phosphorylation of a 38 kDa protein (p38) from rat liver plasma membrane is stimulated by *ras* or endogenous G-proteins. We have now estimated the level of expression of p38 in liver tissues from embryos at different stages of development, regenerating liver and also in tumor cell lines of hepatic origin. Our results indicate that the expression of p38 is negatively correlated with cell division. It is suggested that the phosphorylation of p38, an event which is regulated by *ras* proteins and G-proteins, could be involved in signal transduction processes associated with the inhibitory regulation of cell division.

Cell division; *ras* Protein; Protein phosphorylation; Level of p38

1. INTRODUCTION

One of the mechanisms of regulation of signal transduction across the plasma membrane is protein phosphorylation [1–3]. We had reported earlier [4] a significant increase in phosphorylation of a 38 kDa protein (p38) present in rat liver plasma membranes, by *ras* proteins. Further we showed that glucagon also stimulates the phosphorylation of p38 in an identical manner to *ras*-stimulated phosphorylation. The stimulation of phosphorylation by glucagon was found to be dependent on the presence of guanine nucleotides in the reactions. On the basis of this and other observations, we had suggested the involvement of (a) G protein(s) in p38 phosphorylation [5]. G proteins have been shown to participate in signal transduction pathways including modulation of phosphorylation of cellular proteins through cascades involving second messengers [6]. *ras* proteins also have been implicated to participate in signal transduction pathways on the basis of their GTPase activity and ability to interact with GTPase-activating proteins [7–11]. It has also been shown that *ras* proteins activate MAP kinases [12,13] and regulate K^+ channel in atriocytes [14]. Considering the acid-labile nature of p38 phosphorylation, there exists the possibility that the phosphorylation of p38 represents a step in a hitherto undiscovered mode of signal transduction by G-proteins or *ras*-like proteins. In order to gain greater understanding of the function of p38, we examined its expression in liver cells, liver tumour cell lines and in regenerating liver by using antibodies specific to p38. Our results show that the expression of p38 is negatively correlated with the state of cell division.

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2. MATERIALS AND METHODS

2.1. *Cells and plasma membranes*

Plasma membranes were prepared from adult-, regenerating-, and fetal-rat livers by the method described by Lesko et al. [15]. The plasma membranes prepared by this method have been shown to be highly pure and free from contamination by other cellular constituents [16]. In the case of regenerating livers the cells were collected 48 h after partial hepatectomy. Human liver samples were obtained from local hospitals. Protein estimation was done according to Lowry et al. [17].

2.2. *Phosphorylation reaction*

Phosphorylation reactions were performed in 100 μl reaction mixtures containing 1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid) at pH 7.5, 0.21 M D-mannitol, 0.07 M sucrose, 2.5 mM $MgCl_2$ and 20 μg liver plasma membrane proteins with or without guanine-nucleotides as specified in tables and legends to the figures. The reactions were started by adding 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol) and were terminated by the addition of 50 μl of electrophoretic sample buffer (final concentration of 0.0625 M Tris (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol and 0.001% Bromophenol blue). The samples were then boiled at 100°C for 5 min and analyzed by discontinuous SDS gel electrophoresis with 10% acrylamide in the resolving gel [18]. The gels were fixed in a solution containing 10% isopropyl alcohol and 10 mM sodium pyrophosphate, washed several times with the same solution, dried and autoradiographed. The intensities of the phosphorylated p38-band in different experiments were measured by using a soft laser scanning densitometer (Biomed Instruments, CA) and quantifications made as described previously [19].

2.3. *Antiserum against p38*

p38 was purified from SDS-gels by electroelution [20]. Pure p38 was emulsified in complete Freund's adjuvant. New Zealand White rabbits were injected intradermally with the emulsion at multiple sites on the

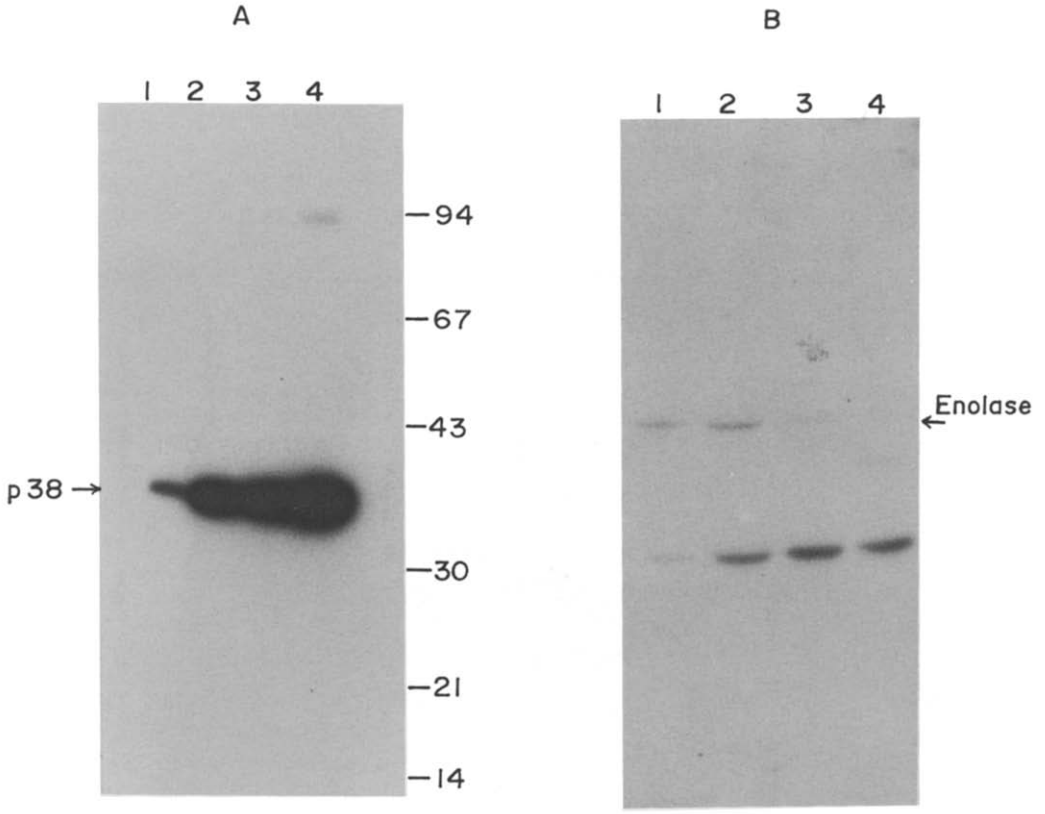

Fig. 1. (A) Expression of p38 at different stages of rat liver development detected by using anti-p38 antibodies. Lane 1, fetal liver (10 days); lane 2, fetal liver (18 days); lane 3, liver from a 10-day-old pup; lane 4, liver from an adult animal. (B) Same blot treated with anti-enolase antibody. Equal protein (100 μg) was loaded in all lanes.

back. Booster doses were given in incomplete Freund's adjuvant at 15-day intervals until positive reaction to p38 was seen on Ouchterlony double diffusion test [21]. The antiserum thus raised recognized both native and phosphorylated p38.

2.4. *Western blots*

Proteins from SDS gels were transferred on to nitrocellulose paper as described [22]. Each time the efficiency of transfer was checked by staining a strip cut from the nitrocellulose paper by Amido black and also by staining the gels with Coomassie blue after transfer. After the transfer the blots were rinsed with phosphate-buffered saline (PBS), incubated for 15 min in buffer A (PBS; 0.25% gelatin, 0.1% Tween 20, 0.01% sodium azide) followed by further incubation for 3 h in buffer A containing 1% horse serum. After three washings with buffer A the blots were incubated overnight with anti-p38 antibody (1:100 made in buffer A) in plastic bags. Then the blots were washed five times with buffer A before incubating overnight with [^{125}I]protein A in buffer A (specific activity 10 μCi/μg with 1 μCi/ml of protein A in buffer A). Then the blots were successively washed over a period of 4 h, five times each with buffer A, buffer A containing 0.4% sarcosyl, buffer A containing additional 150 mM NaCl and again 5 times with buffer A. The blots were then air-dried and exposed to X-ray film (Konica) overnight at −70°C unless otherwise stated. The intensities of the p38-band were measured by using a soft laser scanning densitometer (Biomed instruments, CA) and quantifications made as described earlier [18].

3. RESULTS

The observation that G-proteins and *ras* proteins stimulated the phosphorylation of p38 and the possibility that the phosphorylation could play a role in signal transduction prompted us to examine the presence of the protein in dividing and resting liver cells. It was

Table I

Expression of p38 in liver tissues at different stages of development and in liver tumors[a,b]

Tissues/Tumors		Extent of expression of p38
(I)	*Rat liver*	
(A)	Adult rat liver (Wistar)	100
	Fetal liver (12 days)	11
	Regenerating liver	40
(B)	Normal adult rat liver (F344)	100
	Ciprofibrate-induced liver tumor	14
	Aflatoxin-induced liver tumor	12
	AAF-induced liver tumor	11
(II)	*Human liver*	
	Adult human liver	100
	Fetal liver (4 weeks)	2
	Fetal liver (5 weeks)	10
	Fetal liver (6 weeks)	18
	Fetal liver (28 weeks)	53

[a] 100 μg total protein was taken in each sample.
[b] In each case the extent of expression of p38 in the adult was taken as 100.

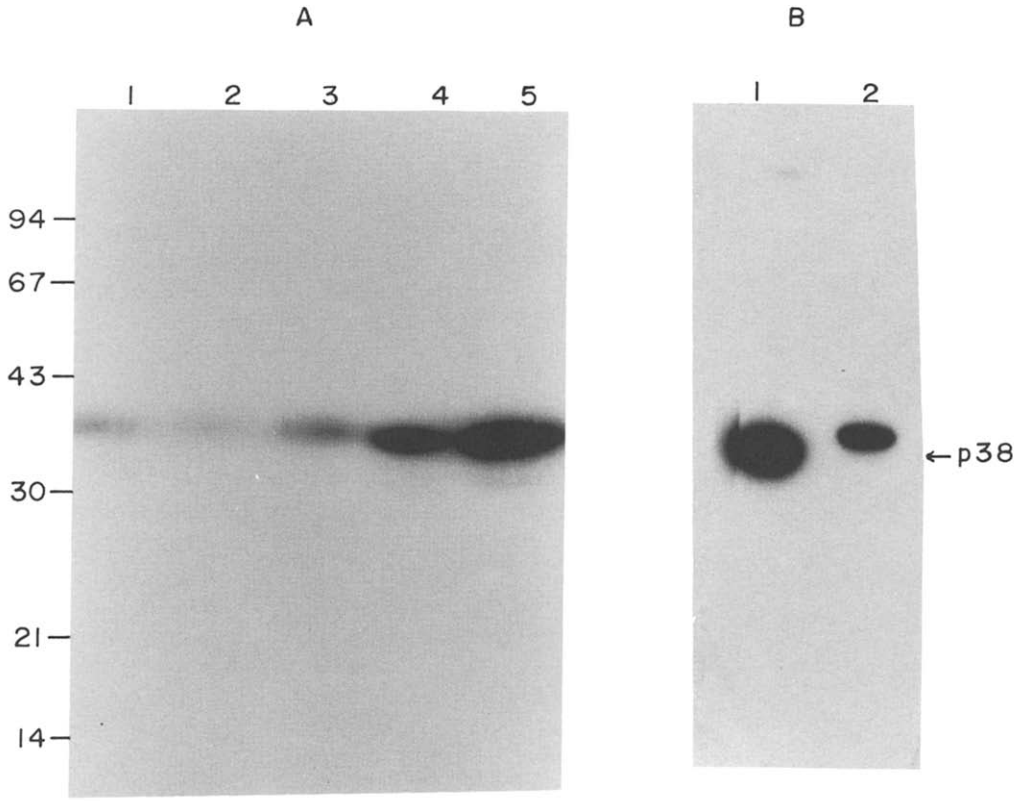

Fig. 2. Expression of p38 in human liver at different stages of development. Lane 1, fetal liver (4 weeks); lane 2, fetal liver (5 weeks); lane 3, fetal liver (6 weeks); lane 4 fetal liver (28 weeks); lane 5, adult human liver. (B) Comparison of relative mobilities of rat p38 (lane 1) with human (adult) p38 (lane 2). 100 μg total protein was used in all lanes.

found that the highest level of expression was in adult rat liver, followed by regenerating rat liver and least in fetal rat liver (Table I). It was also observed that as a function of the stage of development of the liver the expression of p38 gradually increased (Fig. 1). Similar results were obtained from human fetal liver at various stages of development (Fig. 2, Table I). In addition, we carried out partial hepatectomy on adult rats and obtained a dividing population of liver cells after 48 h. Also portions of completely regenerated liver were collected from animals 18 days after partial hepatectomy, rich in non-dividing cells like that of normal liver from the adult animal. It can be seen from Fig. 3 that the expression of p38 was more in completely regenerated liver as opposed to actively regenerating liver. In experiments mentioned above, control Western blot experiments were carried out with antibodies against enolase (kindly provided by Tony Hunter), it was observed that the signal did not follow any particular pattern in various stages of liver development (Fig. 1). This was also the case when the homogenates from regenerating liver were compared with that of normal adult rat liver (data not shown). This observation rules out the possibility of any experimental artefact or generalized effect that could have resulted in difference in signals detected with anti-p38 antibody. Hence one could be certain that the observations were specific to p38.

If the expression of p38 is indeed negatively correlated with the state of cell division one should expect low-expression of p38 in rat liver tumors compared to normal liver. Three liver tumors (kindly provided by J.K. Reddy) which were induced in F344 rats by ciprofibrate, aflatoxin and 2-acetyl aminofluorene (AAF) [23] were tested. Normal livers from F344 rats were used as control for this study. As can be seen from Fig. 4A and Table I, the level of p38-expression was significantly low in all the three tumors compared to that in normal liver. When we probed a parallel blot with anti-enolase antibodies the pattern was entirely different. Normal liver cells and the tumors expressed comparable levels of enolase. In one of the tumors the expression of enolase was higher than the rest (Fig. 4B).

4. DISCUSSION

We have presented evidence to show the negative correlation of p38 expression with liver cell division.

Quantitative studies were carried out using liver tissues of rat and human origin. The results, in addition to confirming the low level of p38 fetal livers compared

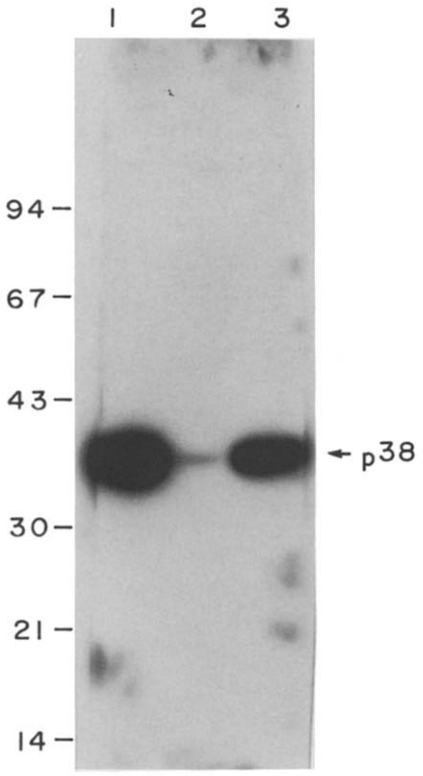

Fig. 3. Expression of p38 in normal adult and regenerating rat liver. The figure shows Western blot with anti-p38 antibodies of homogenates from adult rat liver (lane 1), regenerating rat liver (lane 2) and liver 18 days after partial hepatectomy (lane 3). All the lanes had 100 μg total protein.

to adult liver showed that the expression of p38 was low in regenerating liver; the expression of p38 increased after complete regeneration as opposed to actively regenerating liver. Also expression of p38 was quite low in liver tumors as compared to normal liver. On the other hand, the quantity of enolase did not follow the same pattern in the above-mentioned cases showing thereby that the variations in the level of expression of p38 was not a consequence of any generalized variations in the system examined. The experimental observations would indicate a negative correlation of expression of p38 with the state of cell division. The observation raises the possibility that p38 might be a transducer of signal(s) involved in the negative control of cell proliferation. It is interesting to note that a gene coding for a protein like p21 *ras* has been shown to be able to suppress the transformed phenotype of *ras*-transformed cells [24]. Also it is becoming increasingly clear that the growth arrested state of the cell is an actively maintained process. The expression of several genes has been shown to be increased in growth-arrested state of cells [25]. Recently it has been shown that overexpression of one of the growth-arrest-specific (GAS) genes, *gas*1 inhibits serum-induced transition from the G_0 to the S phase of the cell cycle; ectopic expression of this protein leads to inhibition of DNA synthesis in normal and transformed NIH 3T3 cells [26]. It is interesting to note that p38 is a plasma membrane protein like *gas*1. Another protein, named prohibitin, has also been shown

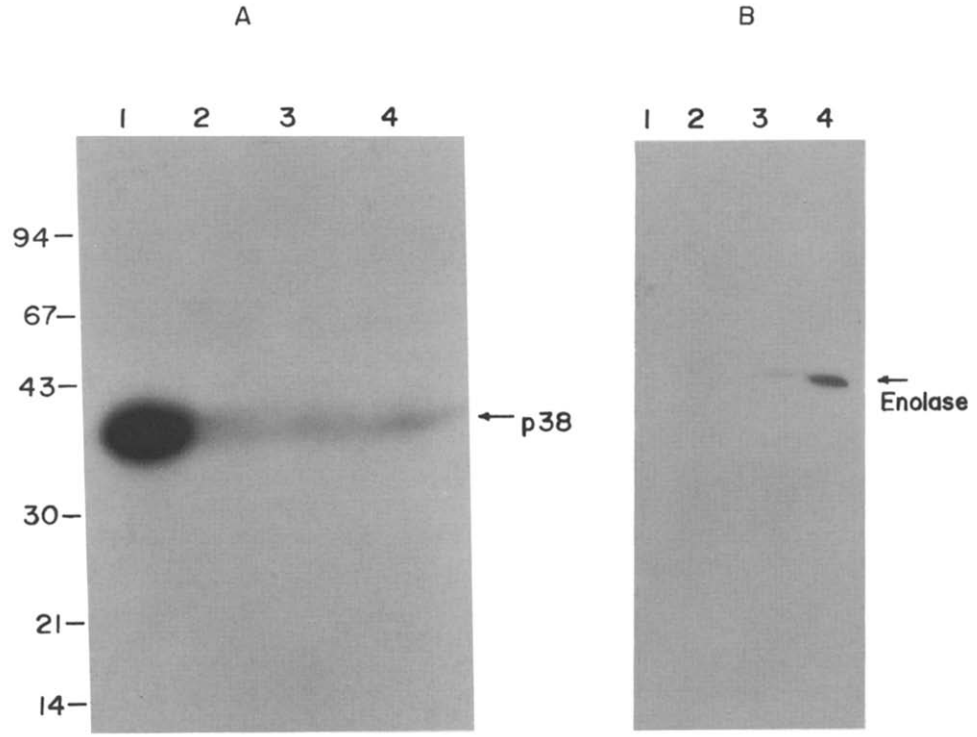

Fig. 4. Expression of p38 in rat liver tumors. (A) Lane 1, normal rat liver (F344) (see text); lane 2, ciprofibrate-induced tumor; lane 3, aflatoxin induced tumor; lane 4, 2-acetyl amino fluorene-induced tumor. In all the experiments 100 μg total protein was loaded in each well and Western blot experiments were performed as described in the text. (B) Parallel blot probed with anti-enolase antibodies.

to block DNA synthesis in normal fibroblasts and HeLa cells [27]. It is possible that the gene encoding p38 is one of the GAS genes. However this remains to be established. In this context it is relevant to note that the proteins encoded by v-*ras* are less efficient in phosphorylation as compared to those coded by c-*ras* genes [4].

It may be noted that other than liver, tissues such as kidney show high levels of p38 (data not shown), although the protein is not present ubiquitously in all the non-dividing tissues. It is therefore unlikely that p38 is likely to be a differentiation marker for hepatocytes.

Since the phosphorylation of p38 is enhanced by *ras* and G-proteins and it occurs in vivo [5], it is possible that the function of p38 in cell division is modulated by phosphorylation. Experiments are currently under way to examine the exact role of p38 in liver cell division and its modulation by phosphorylation.

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