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ras proteins enhance the phosphorylation of a 38 kDa protein (p38) in rat liver plasma membrane

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Phosphorylation of a 38 kDa protein (p38) present in rat liver plasma membrane has been shown for the first time to be enhanced by *ras* proteins. This increase in phosphorylation is about 3–16-fold depending on the incubation time and the type of *ras* protein used. Acid treatment removes phosphate from this protein suggesting that the phosphorylation involves phosphoramidate derivatives of basic amino acids. Experiments carried out in the presence of diethylpyrocarbonate suggest that the phosphorylation occurs on (a) histidine residue(s). It is probable that the function of p38 in the cell is modulated by *ras* proteins through phosphorylation. The significance of phosphorylation of p38 in terms of malignant transformation is presently unknown.

38 kDa protein; *ras* protein; Phosphorylation; (Rat liver plasma membrane)

1. INTRODUCTION

The oncogenes belonging to the *ras* family code for proteins of $M_r \sim 21\,000$ (p21) [1–3]. The precise biochemical function of these proteins in the cell and their role in malignant transformation are not yet fully understood. But they are known to bind GTP and GDP and have GTPase activity [4–6]. Recently there have been reports of the involvement of these proteins in the modulation of phosphorylation of certain mitochondrial membrane proteins [7,8]. Evidence gathered by subcellular fractionation and electron microscopy has shown that both normal and transforming *ras* proteins are present at the inner surface of the plasma membrane [9,10]. If *ras* proteins have (a) biological function(s) in addition to, or apart from their GTP-binding properties and consequential role in signal transduction initiated by this mechanism, the target proteins are likely to be in the plasma membrane, the natural location of the p21 proteins. Since phosphorylation is one of the most common post-translational modifications by which the functions of proteins are modulated, we sought to check if *ras* proteins affect the phosphorylation of any plasma membrane proteins. In this communication we report for the first time evidence for enhancement in phosphorylation of a 38 kDa protein present in rat liver plasma membrane by p21 *ras* proteins.

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2. MATERIALS AND METHODS

The *ras* proteins RAS1[11], EC[12], EC/v-Ha[12] and EJ/v-Ha[12] expressed in *Escherichia coli* and purified to homogeneity were provided by M. Poe, R.B. Stein and J.B. Gibbs of Merck Sharp and Dohme Research Laboratories.

Plasma membranes were isolated from livers of male Wistar rats according to Lesko et al. [13]. The mitochondrial fraction was obtained from rat liver as described by Greenawalt [14] and was further purified by the procedure of Bogenhagen and Clayton [15]. Solubilization by glucose 6-phosphate and phosphorylation of proteins from mito-

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chondria were carried out as reported by Backer and Weinstein [8].

The phosphorylation reactions which contained rat liver plasma membrane proteins were performed at room temperature in a 100 μl reaction mixture comprising 1 mM Hepes 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 *ras* protein (at different concentrations as shown in results) or GDP at various concentrations (50–2500 nM) as required for individual experiments. The reactions were started by adding 10 μCi [γ-^{32}P] ATP (3000 Ci/mmol) and were terminated with the addition of 50 μl sample buffer (to give final concentrations of 0.0625 M Tris, pH 6.8, 2% SDS, 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 [16] with 10% acrylamide in the resolving gel. 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. In order to determine the lability of the phosphate moieties, gels were treated overnight with a solution containing 15% (v/v) acetic acid and 15% (v/v) methanol. Staining and destaining of the gels were accomplished during the same period by including 0.2% Coomassie blue in the solution described above for the first 2 h. The relative intensities of the bands on the autoradiograms were measured by using a soft laser scanning densitometer (Biomed Instruments, CA).

The proteins were transferred from gels to nitrocellulose paper according to the method described by Towbin et al. [17] and the blots were probed with antibodies using the procedure described by Gould et al. [18] with minor modifications.

3. RESULTS

The phosphorylation of rat liver plasma membrane was carried out in the presence of four *ras* proteins viz., RAS1 (The 21 kDa protein encoded by the *ras* oncogene of Harvey murine sarcoma virus with Arg-12, Thr-59 and Gln-61) [11], EC (normal human c-Ha-ras-1 with Gly-12, Ala-59 and Gln-61) [12], EC/v-Ha (EC containing 3′

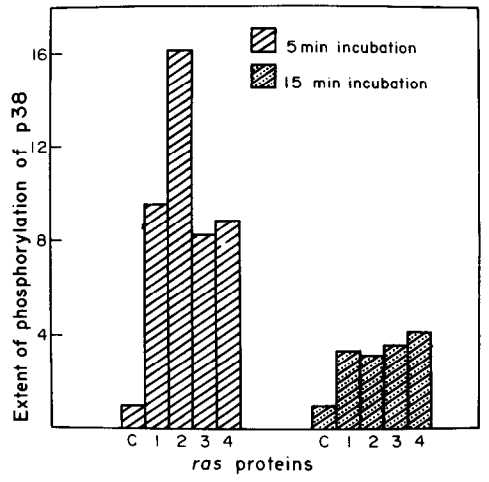

Fig.1. Extent of enhancement in phosphorylation of p38 by *ras* proteins. Control value is taken as unity. The phosphorylation reactions were performed for 5 min or 15 min. (Columns: C, Control; 1, RAS1; 2, EC; 3, EC/v-Ha; 4, EJ/v-Ha.

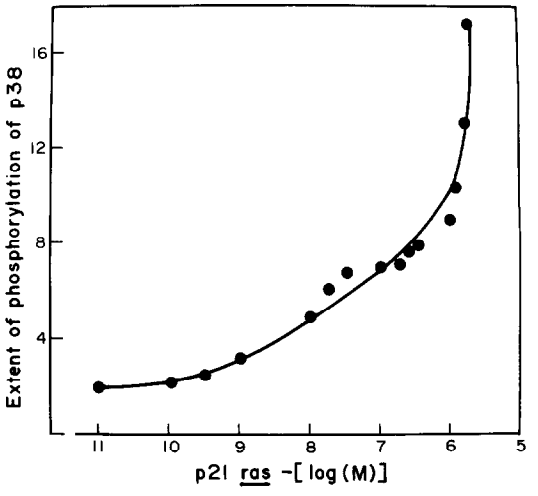

Fig.2. Extent of enhancement in phosphorylation of p38 with increasing concentrations of p21 *ras*. Liver plasma membranes (20 μg) were incubated with various concentrations of EC (10^{+11} M to 2.5×10^{-6} M) and the phosphorylation reactions were performed as described in section 2. Following the autoradiography the p38 band was removed from the dried gel and counted for radioactivity. Values are expressed relative to control. Control value is taken as unity.

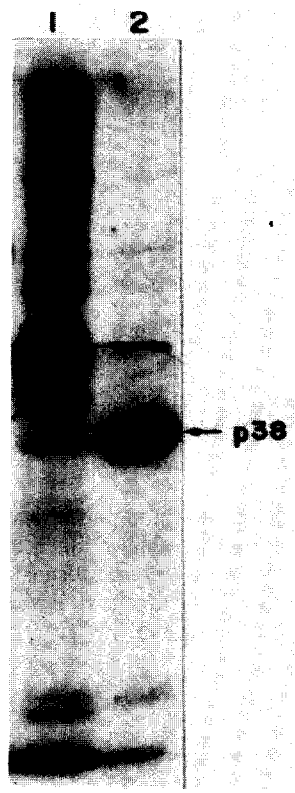

Fig.3. Enhancement in phosphorylation of 38 kDa protein (p38) by *ras* protein. Phosphorylation reactions were carried out for 5 min as described in section 2. Lanes: 1, liver plasma membrane, control; 2, liver plasma membrane with RAS1.

coding region of viral Harvey *ras* with Gly-12, Thr-59 and Gln-61) [12], and EJ/v-Ha (oncogenically activated human c-Ha-ras-1 containing 3′ coding region of viral Harvey *ras* with Val-12, Thr-59 and Gln-61) [12]. With all the *ras* variants tested, a large increase in phosphorylation of a 38 kDa protein (p38) was observed (figs 1 and 3). When the reaction mixture was incubated for 5 min, this increase was 8–16-fold and with 15 min incubation time the increase was 3–4-fold over control (fig.1). Further, enhancement in phosphorylation of p38 was tested at various concentrations of EC (10 pM–2500 nM). Even at the lowest concentration of *ras* there was slight enhancement in phosphorylation of p38 (fig.2).

In order to check the nature of phosphorylation the gels were treated with acid prior to autoradiography (see section 2). It was observed that almost all of the ^{32}P incorporated into p38 was removed by acid treatment (fig.4). This suggested that the phosphorylation occurred on one or more of the basic amino acids such as arginine, histidine and lysine. Furthermore, when the phosphorylation was carried out in the presence of 10 mM diethylpyrocarbonate (DEP) the phosphorylation of p38 was completely inhibited in the control sample as well as in samples with RAS1, EC, EV/v-Ha and GDP (500 nM–2500 nM) suggesting the possible site(s) of phosphorylation to be (a) histidine residue(s) (fig.5).

All the four variants of *ras* used in our study are 1:1 binary GDP complexes [19]. GDP binds to these proteins with a dissociation constant of about 10^8 M^{-1} [20]. The expected dissociation of bound GDP at the concentration of *ras* proteins used in our experiments (micromolar) would be 5–20%. In order to check whether GDP alone had any effect on the phosphorylation of p38, phosphorylation reactions were carried out using different concentrations of GDP. The range of concentrations covered the levels of free GDP expected and the total GDP associated with the *ras* proteins. It was observed that GDP also enhanced the phosphorylation of p38 (table 1). The increase at 5 min incubation was higher than that observed at 15 min at all the different concentrations of GDP just as with the *ras* proteins. But there were clear-cut quantitative differences in the enhancements observed with GDP (from 50–2500 nM) and *ras* proteins. The increase in phosphoryla-

Table 1

Effect of GDP on the phosphorylation of p38

Concentration of GDP (nM)	Extent of enhancement in phosphorylation of p38 (% of control)	
	5 min[a]	15 min[a]
0	100	100
50	186	101
100	238	102
500	397	143
1000	438	175
2000	463	199
2500	524	204

[a] Phosphorylation reactions were performed as described in section 2 for 5 min or 15 min as stated

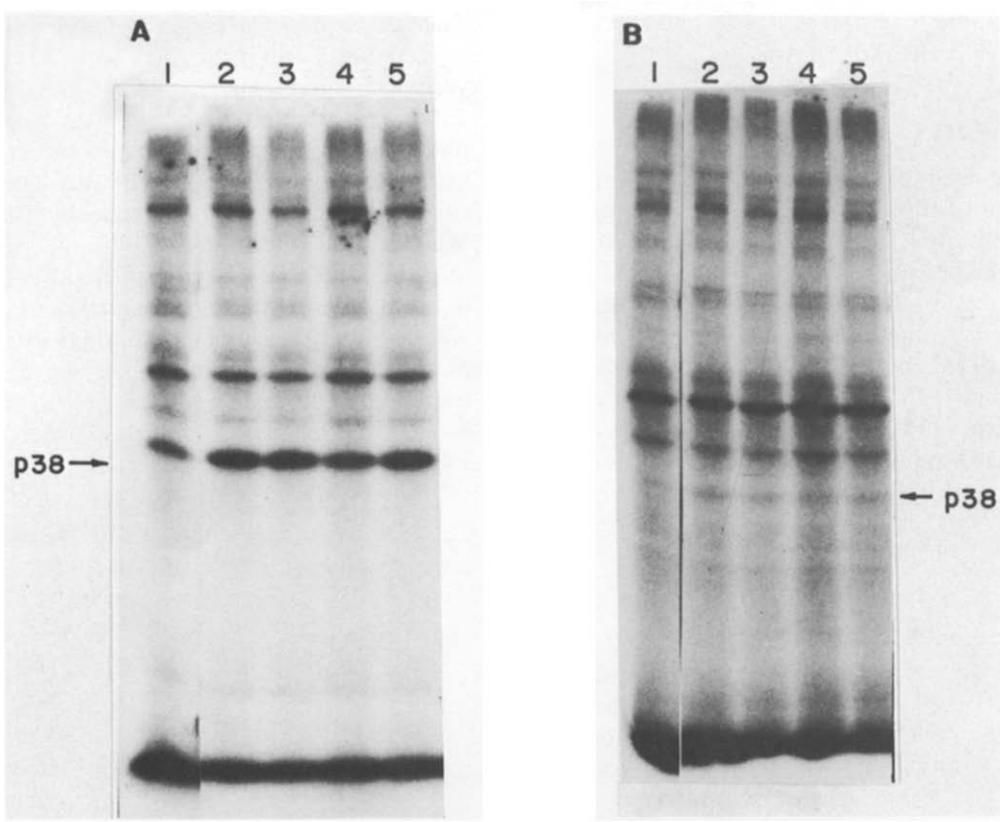

Fig.4. Effect of acid treatment on phosphorylation of p38. Phosphorylation reaction mixtures were incubated for 15 min and the gels were autoradiographed with (B) or without (A) acid treatment. Lanes: 1, control; 2, RAS1; 3, EC/v-Ha; 4, EC; 5, EJ/v-Ha.

tion of p38 with *ras* protein was at least 2-fold higher than the increase brought about by the highest possible concentration of GDP expected to be present in association with the *ras* proteins.

In order to ascertain whether the phosphorylation of p38 that we have observed in liver plasma membrane is distinct from the phosphorylated proteins of the mitochondria reported by Backer and Weinstein [8], a comparison between the effect of *ras* proteins on the phosphorylation of liver plasma membrane proteins and mitochondrial membrane proteins was made under identical conditions. These results are presented in fig.6. Our results confirm the observations of Backer and Weinstein in demonstrating increased phosphorylation of a 36 kDa protein in mitochondrial membranes. But, in addition, it can be clearly seen that in the plasma membrane preparations from liver tissues the protein that shows increased phosphorylation in the presence of *ras* proteins is distinct in molecular mass (38 kDa) from the one (36 kDa) reported by Backer and Weinstein. These authors have also reported that *ras* proteins inhibit the phosphorylation of a 17 kDa protein in mitochondrial membranes. However, with plasma membrane proteins *ras* proteins did not bring about any changes in the extent of phosphorylation of any protein other than p38.

In order to check whether the p38 is different from or similar to the transformation associated 34–39 kDa (p36) protein reported in various systems [18] Western blot experiments were done with anti-p36 antibodies raised against p36, the substrate of $pp60^{src}$ from chicken embryo fibroblasts (kindly provided by Dr G.S. Martin, University of California) and antiserum raised against p36 from Zajdela ascitic hepatoma (a chemically induced rat liver tumour). Neither of

these antibodies cross-reacted with p38 from liver plasma membrane (not shown).

4. DISCUSSION

We have demonstrated that *ras* proteins enhance the phosphorylation of a 38 kDa protein in rat liver plasma membrane (p38). We have also shown that this protein is distinct from the p36 reported to be present in the plasma membranes of various tumours and normal tissues [18]. Further, we have established that the p38 from liver plasma membrane is different from the mitochondrial membrane protein (36 kDa) whose phosphorylation has been reported to be increased by *ras* proteins [7,8].

The enhancements in phosphorylation of p38 observed at 5 min incubation with all the four *ras* variants are higher compared to those observed at 15 min incubation time. A similar decrease in phosphorylation of p38 at 15 min as against 5 min incubation was seen with GDP also. Qualitatively similar observations were made by Backer and Weinstein [8] in the case of phosphorylation of a mitochondrial membrane protein. It is likely that in both cases the observed reduction in phosphorylation with time could be resulting from probable protein phosphatase activity present in the membrane preparations.

The experiments with various concentrations of EC showed that the *ras* protein is effective in

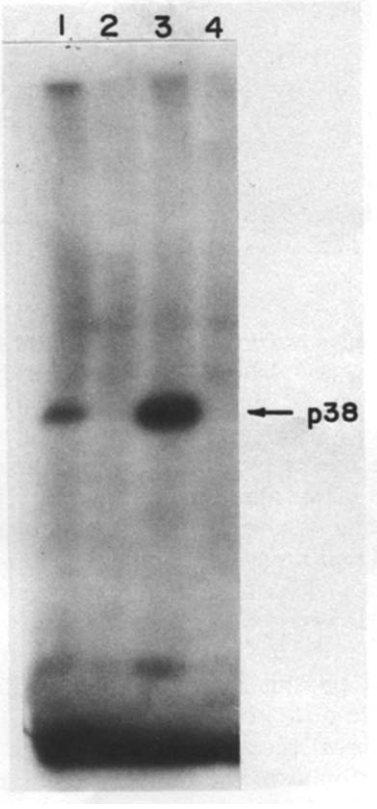

Fig.5. Diethylpyrocarbonate (DEP) inhibits phosphorylation of p38. Phosphorylation reactions were carried out for 5 min as described in section 2 with or without 10 mM DEP. Lanes: 1, liver plasma membrane, control; 2, liver plasma membrane with DEP; 3, liver plasma membrane, with RAS1, control; 4, liver plasma membrane with RAS1 and DEP.

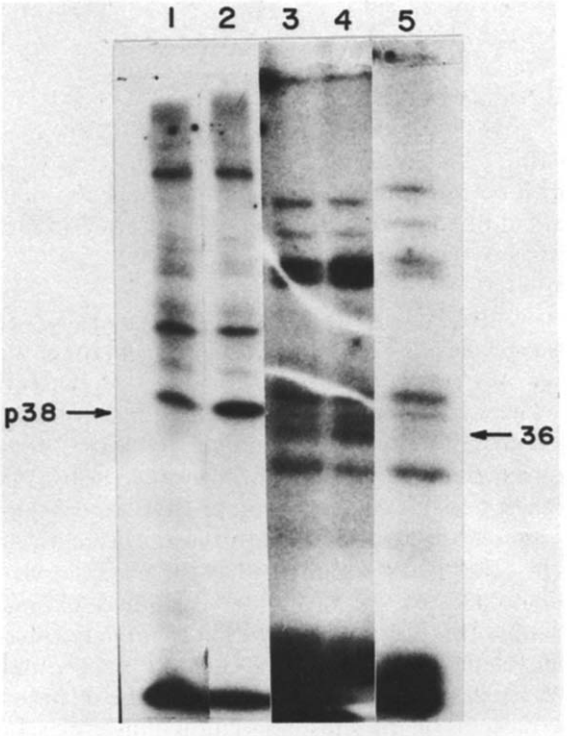

Fig.6. Comparison of phosphorylation patterns of rat liver plasma membrane proteins with that of mitochondrial membrane proteins. Lanes: 1, liver plasma membrane, control; 2, liver plasma membrane with RAS1; 3, mitochondrial membrane proteins, control; 4, mitochondrial membrane proteins with RAS1; 5, mitochondrial membrane proteins with RAS1 treated with acid prior to autoradiography. The positions of p38 from liver plasma membrane and 36 kDa protein from mitochondrial membrane are indicated.

bringing about the enhancement in phosphorylation of p38 even at the lowest concentration tested (nearly 2-fold over control at 10 pM level). The present experiments were performed keeping the membrane protein concentration constant (20 μg). It may also be noted that the concentrations of p21 *ras* required to bring about increased phosphorylation of p38 could be different in vivo and it would be important to check whether even smaller concentrations of p21 could bring about increased phosphorylation of p38 under these conditions.

Although GDP alone also influences the phosphorylation of p38, our results clearly show that the increase in phosphorylation of p38 brought about by *ras* proteins is higher than that observed with GDP. GDP is associated with *ras* proteins as a complex and even if the maximum dissociation is taken into account, the enhancement obtained at this level (500 nM GDP at the *ras* protein concentrations used in the present experiments, table 1) is 2–4-times less than that is observed with *ras* proteins both at 5 min and 15 min incubation times. Also, even the total GDP present in association with the *ras* protein cannot bring about the same extent of enhancement observed with *ras* proteins. Our results clearly indicate that at concentrations of GDP in excess of the total GDP expected to be associated with *ras* proteins, the enhancement in phosphorylation of p38 is considerably lower than that observed with *ras* proteins (see table 1).

The acid lability of the phosphate incorporated into p38 would argue that the phosphorylation involved phosphoramidates and occurred on basic amino acids such as arginine, histidine and lysine. When the reactions were carried out in the presence of 10 mM diethylpyrocarbonate, the phosphorylation of p38 was completely inhibited (fig. 5). As DEP is a specific inhibitor of histidine phosphorylation [21] our results narrow down the observed phosphorylation of p38 to (a) histidine residue(s). The significance of such a phosphorylation is not clear, although protein kinases that phosphorylate structural proteins on basic amino acids are known [22].

It remains to be seen whether the enhancement in phosphorylation of p38 by *ras* proteins occurs in vivo. Detailed studies of p38 and identification and characterization of the kinase responsible for this phosphorylation are also necessary for understanding the relevance of our observations to transformation-related events. It is also necessary to see if oncogenically activated *ras* proteins act differently on phosphorylation of p38 from normal *ras* proteins. So far, the *ras* proteins are known to have only autokinase activity [12]. Since p38 is found in the plasma membrane where p21 is located, it would be important to determine the possible function of p38 and check whether p21 *ras* modulates the function of p38 by phosphorylation.

Our present observations assume significance in terms of understanding *ras* function in the light of several other observations, despite the fact that we do not know at present the biological significance of the p38 phosphorylation. The work of Finkel et al. [23], Lacal et al. [24] and Der et al. [25] suggest that activation of efficient transforming properties by *ras* p21 proteins can occur by mechanisms independent of variation in GTP binding or GTPase activity. Furthermore, the work of McCormick et al. [26] suggests that the amino acids defining guanine specificity are Asn-116 and Asp-119 and that sequences around amino acid 145 may also contribute to guanine binding. On the other hand, the phosphoryl-binding region is made up of amino acid sequences from 10 to 16 and from 57 to 63 of p21. In the light of these results taken together with the observations that mutations at positions 12, 59 and 61 in c-*ras* p21 are concerned with transforming properties, a situation exists whereby it is not possible to correlate the biologically significant mutation with any structural changes at the GTP-binding region. It is, therefore, important to examine the biological significance of the p38 phosphorylation that we have observed.

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