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Glucagon and p21 *ras* Enhance the Phosphorylation of the Same 38-Kilodalton Membrane Protein from Rat Liver Cells

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We had reported earlier the enhanced phosphorylation of a 38-kilodalton protein (p38) in rat liver plasma membrane by *ras* proteins. Now we show that glucagon increased the phosphorylation of the same protein. The nature and site(s) of phosphorylation were the same as those for the *ras* proteins. Both ATP and GTP could donate phosphate for the phosphorylation of p38. The stimulation of p38 phosphorylation by glucagon was guanine nucleotide dependent. This observation, together with our data on the stimulation of p38 phosphorylation by AlF_4^-, suggest the involvement of G proteins in the reaction. We also showed that glucagon stimulates the phosphorylation of p38 in vivo.

The ubiquitous occurrence of the *ras* family of genes in eucaryotic organisms (2) and evidence for their expression in a number of human (9, 36, 38, 41) and animal (1, 2, 5, 19, 45) tumors as well as normal tissues (13, 32, 42) has resulted in considerable recent interest in the study of *ras* oncogenes. Although the *ras* proteins are known to bind GTP and GDP and have GTPase activity (16, 31), the biological function of *ras* proteins remains unknown. GTPase-activating proteins have been shown to interact with *ras* proteins (31), but the exact role of *ras* in signal transduction still remains to be understood (24). Since phosphorylation is known to play a key role in signal transduction (7, 10, 22, 34), we had investigated the effect of *ras* proteins on the phosphorylation of plasma membrane proteins from rat liver and had reported increased phosphorylation of a 38-kilodalton protein (p38) present in the preparations (20).

The *ras* proteins used in our experiments contained bound GDP (37). We had observed also (20) that GDP stimulates the phosphorylation of p38, although to a lesser extent compared with the stimulation observed in the presence of *ras* proteins. These observations suggested possible involvement of guanine nucleotides in the phosphorylation of p38. It was therefore of interest to check whether any of the factors which act on rat liver cells in a guanine nucleotide-dependent manner affected the phosphorylation of p38. We report here the effect of glucagon on the phosphorylation of p38 and show for the first time that the *ras* gene product and glucagon stimulate the phosphorylation of the target protein (p38) at an identical site(s).

MATERIALS AND METHODS

***ras* protein.** The *ras* protein EC (normal human c-Ha-*ras*-1 with Gly-12, Ala-59, and Gln-61) expressed in *Escherichia coli* and purified to homogeneity (15) was provided by J. B. Gibbs of Merck Sharp & Dohme Research Laboratories.

Plasma membrane preparations. Plasma membranes were isolated from livers of male Wistar rats by the procedure of Lesko et al. (27). This method yields highly purified plasma membrane preparations as judged by marker enzyme assays (40).

Phosphorylation reactions. The in vitro phosphorylation reactions were performed as follows. To a 100-μl reaction mixture, 0.21 M D-mannitol, 0.07 M sucrose, 1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) at pH 7.5, 2.5 mM $MgCl_2$, and 20 μg of liver plasma membranes were added. The reactions with or without glucagon or *ras* (EC) were started by adding 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol), and after incubation (at 30°C) for 5 min, they were terminated by the addition of 50 μl of sample buffer (to give a final concentration of 0.0625 M Tris [pH 6.8], 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue). In the experiments involving labeled GTP, the reactions were started with 1 μM [γ-^{32}P] GTP (>10 mCi/mmol; Amersham International plc., Buckinghamshire, England). The reaction mixtures, after the addition of sample buffer, were boiled at 100°C and analyzed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (26) with 10% acrylamide in the resolving gel. After electrophoresis, the gels were treated with a solution containing 10% isopropyl alcohol and 10 mM sodium pyrophosphate, washed several times with the same solution, dried, and autoradiographed. The acid lability of the phosphate moieties was checked by treating the gels with a solution containing 15% (vol/vol) acetic acid and 15% (vol/vol) methanol. The relative intensities were measured by using a soft laser scanning densitometer (Biomed Instruments, Inc., Fullerton, Calif.), and quantification was done as described previously (29).

The stimulation by AlF_4^- was done by the method of Sternweis and Gilman (43). The liver plasma membrane was incubated with 20 μM $AlCl_3$ plus 5 mM NaF for 50 min at 20°C alone or in combination with either GDP or *ras* before phosphorylation.

Peptide mapping. After electrophoretic separation and autoradiography, the phosphorylated p38 band was excised from the dried gel, cut into small pieces, and subjected to peptide mapping with *Staphylococcus aureus* V8 protease (Boehringer GmbH, Mannheim, Federal Republic of Germany) as described by Cleveland et al. (6). The resolving gel had 15% acrylamide. After electrophoresis, 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.

Antiserum to p38. Pure p38 was obtained by electroelution (21) and was dissolved in phosphate-buffered saline and emulsified in complete Freund adjuvant and injected (100 μg of protein) intradermally into multiple sites on the back of a New Zealand White rabbit. Booster doses were given in

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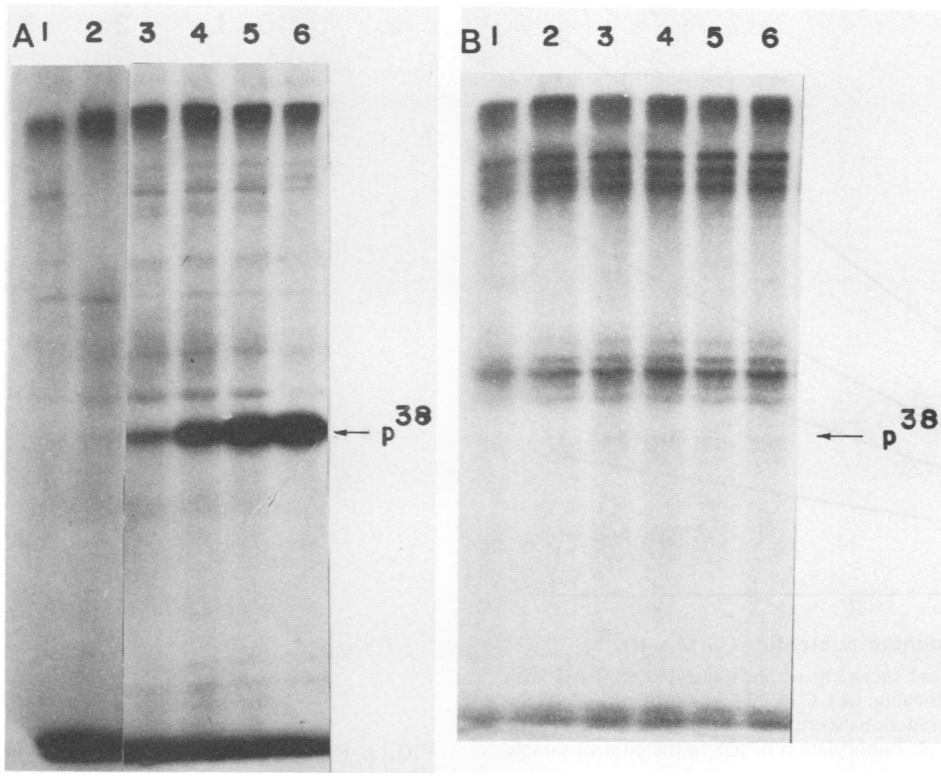

FIG. 1. Enhancement of phosphorylation of p38 by glucagon (20 μg/ml). Phosphorylation reactions were performed as described in the text. (A) Lanes: 1, control; 2, glucagon; 3, glucagon plus 10 nM GTP; 4, glucagon plus 100 nM GTP; 5, glucagon plus 500 nM GTP; 6, *ras* (EC) (20 μg/ml). Exposure time was 12 h at −70°C with intensifying screens. (B) Autoradiography after acid treatment of the gel. Lanes and exposure conditions are the same as in panel A.

incomplete Freund adjuvant. Test bleedings were monitored by the Ouchterlony double-diffusion technique (35).

Immunoprecipitation. The products of in vitro phosphorylation were immunoprecipitated as follows. Phosphorylation reactions were done as described above. After incubation for 5 min at 30°C, reactions were terminated by the addition of 50 μl of 3× immunoprecipitation buffer (1× buffer is 10 mM Tris [pH 7.4], 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 2 mM EDTA, and 0.15 M NaCl) and rapid cooling in crushed ice. Immunoprecipitation reactions were performed essentially by the procedure described by Davis et al. (8). After immunoprecipitation followed by electrophoresis, the gels were fixed and washed with a solution containing 10% isopropyl alcohol and 10 mM sodium pyrophosphate before drying and autoradiography.

In vivo phosphorylation. Liver cells were grown in suspension (3, 14) in phosphate-free Dulbecco modified Eagle medium (10^6 cells per ml containing 1 mCi of carrier-free ^{32}P per ml). The cells were incubated at 37°C for 90 min to label the intracellular nucleotides. At the end of the incubation, glucagon (20 μg/ml) was added and the cells were further incubated for 5 min. The incubation was terminated by rapid cooling in crushed ice, and the cells were immediately collected by centrifugation and lysed in immunoprecipitation buffer and the immunoprecipitation experiments were performed as described above.

Other procedures. Protein was estimated by the method of Lowry et al. (28). Protein bands from gels were transferred to nitrocellulose papers by the method described by Towbin et al. (47), and the blots were probed with antibodies by the procedure described by Gould et al. (18).

RESULTS

Stimulation of p38 phosphorylation by glucagon. We studied the effect of glucagon on phosphorylation in the presence or absence of GTP or GDP in the reaction mixtures. Glucagon stimulated the phosphorylation of p38 markedly in the presence of GTP (Fig. 1A and 2). There was no increase in phosphorylation of p38 in the absence of GTP (Fig. 1A). The stimulation of phosphorylation brought about by a combination of GTP and glucagon was higher than the stimulation obtained with GTP alone (Fig. 2). When GDP was used in place of GTP, GDP also stimulated the phosphorylation, albeit to a lesser extent than that observed with GTP (Fig. 2).

Nature and sites of p38 phosphorylation. We studied the phosphorylation of p38 in the presence of glucagon or *ras* (EC) in detail. The electrophoretic mobility of p38 phosphorylated in the presence of *ras* or glucagon was found to be the same (Fig. 1A). When we checked for the lability of the phosphate moiety by subjecting the gels to treatment with 15% acetic acid (see Materials and Methods), the phosphate moiety was completely removed (Fig. 1B). Also, we observed that the phosphate moiety on p38 tends to be stable in alkali at 55°C (1 M KOH for 1 h). However, at higher temperatures or with longer incubation time, the phosphate was labile (unpublished data), indicating that the products of p38 phosphorylation perhaps involved acyl phosphates (12). Further, we compared the sites of phosphorylation by peptide mapping (6). The results presented in Fig. 3 show identical peptides of p38 phosphorylated in the presence of *ras* or glucagon.

GTP can be a phosphate donor for p38 phosphorylation.

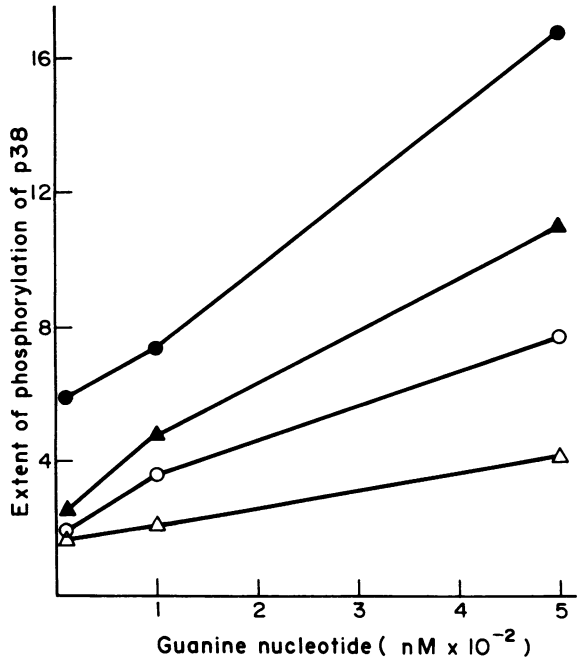

FIG. 2. Extent of enhancement of phosphorylation of p38 with GDP (△), GDP plus glucagon (▲), GTP (○), and GTP plus glucagon (●). Phosphorylation reactions were performed as described in the text. The intensity of phosphorylation of p38 in the control sample was taken as unity.

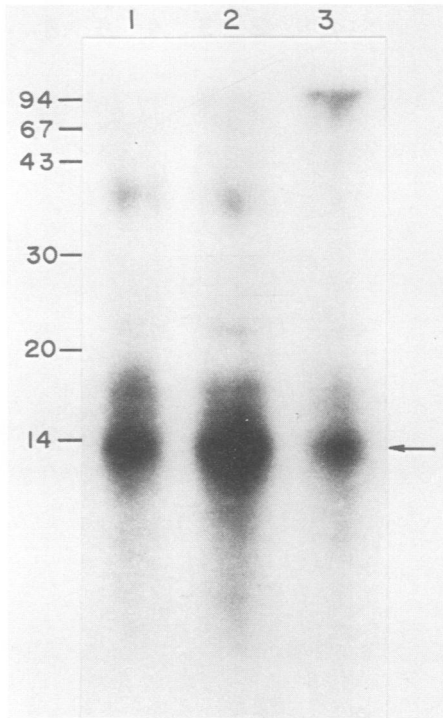

FIG. 3. Peptide mapping of p38. After autoradiography, the p38 band was cut out and the peptide mapping was performed with 5 μg of V8 protease. Lanes: 1, glucagon plus GTP (500 nM); 2, *ras* (EC); 3, glucagon plus GDP (500 nM). The position of the phosphorylated peptide is indicated by an arrow. Molecular size markers (in kilodaltons) are indicated on the left. Exposure time was 3 days at −70°C with intensifying screens.

Initially, we used ATP as the phosphate donor in experiments done to study the stimulation of p38 phosphorylation with glucagon. Since the presence of a guanine nucleotide was obligatory in the reaction mixtures, it was of interest to check whether GTP could be used instead of ATP in the phosphorylation reactions. We used 1 μM [γ-^{32}P]GTP (>10 mCi/mmol) without any cold GTP for these reactions. GTP could donate phosphate for p38 phosphorylation. Also, *ras* and glucagon could enhance phosphorylation of p38 under these conditions as well (Fig. 4A). There exists a theoretical possibility that GTP donated its terminal phosphate to ADP and the ATP thus generated was the phosphate donor. To rule this out, we used [γ-^{32}P]GTP in the presence of a 10-fold excess of cold GTP or GppNHp. Under these conditions, the incorporation of phosphate into p38 was almost totally abolished (data not shown).

Having ascertained that GTP can indeed be a phosphate donor, we did experiments to check whether ATP and GTP donated phosphate via the same reaction. The phosphate incorporated into p38 from ^{32}P-labeled GTP was also acid labile (Fig. 4B). Further, the comparative peptide maps of p38 phosphorylated with ^{32}P-labeled ATP and ^{32}P-labeled GTP showed that the phosphorylation occurred on identical sites (Fig. 5). We determined the apparent K_m for each nucleotide. Cold GDP or GTP (500 nM) was included in the experiments involving labeled ATP, since the presence of guanine nucleotides is obligatory for p38 phosphorylation. ATP alone did not stimulate p38 phosphorylation in the concentration range of 1 nM to 100 μM. The apparent K_m for ATP was 35 nM and that for GTP was 6 μM. In competition experiments in the presence of cold GTP and ^{32}P-labeled ATP or cold ATP and ^{32}P-labeled GTP, each nucleotide inhibited the incorporation of label into p38 from the other. The incorporation of ^{32}P into p38 from labeled ATP (30 nM) was completely abolished at 10 μM cold GTP and was slightly lowered at 5 μM GTP. On the other hand, cold ATP (highly pure, prepared by phosphorylation of adenosine; Sigma Chemical Co., St. Louis, Mo.) abolished incorporation of the label into p38 from ^{32}P-labeled GTP (1 μM) slightly at 50 nM and completely at 500 nM. These data indicate that ATP and GTP contribute phosphate for p38 phosphorylation via similar reaction mechanisms, albeit with different K_ms.

Further, we estimated the stoichiometry of in vitro phosphorylation. The radioactive phosphate incorporated into p38 was 0.3 mol/mol of protein with GTP as the phosphate donor and 0.006 mol/mol of protein when labeled ATP was used for phosphorylation. These estimations represent the phosphorylation of p38 at a minimum level of stimulation, that is, with 1 μM ^{32}P-labeled GTP without any stimulant or with 30 nM ^{32}P-labeled ATP and 500 nM cold GDP. The values for apparent K_m and stoichiometry are to be considered with the caveat that they were determined by a crude system, and the estimation of exact values should await the purification of the p38 phosphorylation system.

Stimulation of p38 phosphorylation by AlF_4^-. The results of experiments on phosphorylation of p38 showed that glucagon, whose action in liver cells is known to be mediated by G proteins (33), stimulated p38 phosphorylation. This stimulation required guanine nucleotides. The combination of glucagon with the guanine nucleotides was much more effective than the guanine nucleotide alone. Also, GTP analogs such as GppNHp stimulated p38 phosphorylation (data not shown). These are hallmarks of G-protein involvement in any biological process (17). We used another crite-

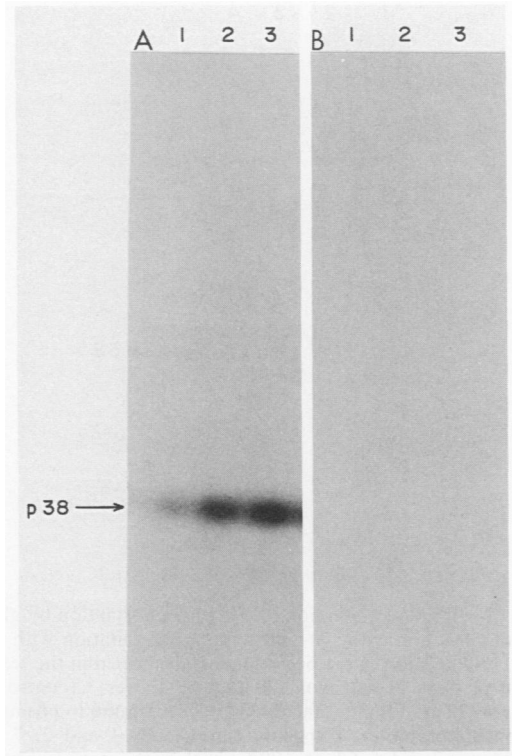

FIG. 4. Phosphorylation of p38 with GTP as the phosphate donor. Phosphorylation reactions were performed as described in the text with 1 μM [γ-^{32}P]GTP (>10 mCi/mmol). (A) Lanes: 1, control; 2, glucagon (20 μg/ml); 3, *ras* (EC) (20 μg/ml). Exposure time was 12 h at −70°C with intensifying screens. (B) Gel treated with acid before autoradiography. Lanes and exposure conditions are the same as in panel A.

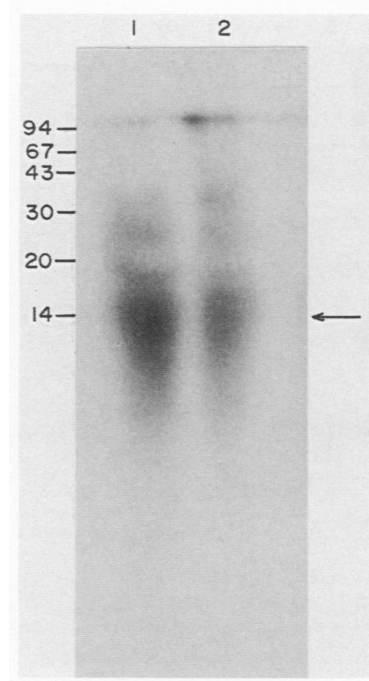

FIG. 5. Comparative peptide mapping of p38 phosphorylated with labeled GTP (lane 1) or ATP (lane 2). The phosphorylation reactions were performed as described in the text with [γ-^{32}P]GTP or [γ-^{32}P]ATP. The p38 band in each case was subjected to peptide mapping with 2 μg of V8 protease. Approximately equal counts were loaded in both the lanes. Exposure time was 5 days at −70°C with intensifying screens. Molecular size markers (in kilodaltons) are indicated on the left. The major phosphopeptide is indicated by an arrow.

rion to ascertain the involvement of G protein(s) (17), namely, the stimulation of phosphorylation by AlF_4^-. AlF_4^- can activate G proteins directly without the involvement of a receptor (17). AlF_4^- has been shown to bind to GDP, and this complex acts like GTP. AlF_4^- when bound with GDP behaves like the gamma phosphate of GTP (4).

When we used AlF_4^- in our system, the combination of GDP and AlF_4^- stimulated the phosphorylation of p38 considerably (Table 1). This effect was more than that observed with GDP alone. Table 1 shows this effect at 1 μM GDP (1 μM GDP was used because 20 μg of *ras* per ml has approximately same amount of bound GDP, see below). We also checked this effect at 0.5 and 2.5 μM GDP. The same results were obtained in those cases as well (data not shown). Also, the results of our experiments showed that AlF_4^- in combination with GDP-bound *ras* was quite effective in stimulating p38 phosphorylation. This effect was more than the effect of *ras* alone (Table 1). AlF_4^- alone did not have any appreciable effect on p38 phosphorylation, showing thereby that it was not due to inhibition of phosphatases or other such effects.

Antiserum to p38. To study the phosphorylation of p38 in vivo, we needed antibodies specific to p38. We electroeluted the p38 band from Coomassie blue-stained gels (21) and raised antibodies against p38 as described above. When the Ouchterlony test showed positive results with pure p38, we checked the specificity of the antibodies by Western blots (immunoblots) (18) of liver plasma membrane of total liver extract. Only p38 was recognized at various dilutions of the antibody (data not shown).

To test whether the antibodies recognized the native and also the phosphorylated forms of p38, we did immunoprecipitations as described in Materials and Methods. At all dilutions, the p38 band was the most prominent one (Fig. 6). At 1:100 dilution, p38 was the only protein that was immunoprecipitated. With preimmune serum, no p38 precipitation was observed (even after long exposure). To make sure that the protein immunoprecipitated was indeed p38, we electrophoresed the products of in vitro phosphorylation reactions on the same gel. The electrophoretic mobility of immunoprecipitated phosphoprotein was the same as that of the protein phosphorylated in vitro (Fig. 6, lanes 5, 6, and 7). It can also be seen from Fig. 6 that although there were many phosphoproteins in the starting material, the antibodies precipitated only p38. Taking all these data together, one

TABLE 1. Effect of AlF_4^- on phosphorylation of p38[a]

Addition	Extent of enhancement in phosphorylation of p38 (% of control)
None	100
AlF_4^-	185
GDP (1 μM)	450
GDP (1 μM) + AlF_4^-	870
ras (EC) (20 μg/ml)	1,175
ras (EC) + AlF_4^- (20 μg/ml)	1,762

[a] The experiment was performed as described in the text. The intensity of the p38 band when no addition was made (control) was taken as 100.

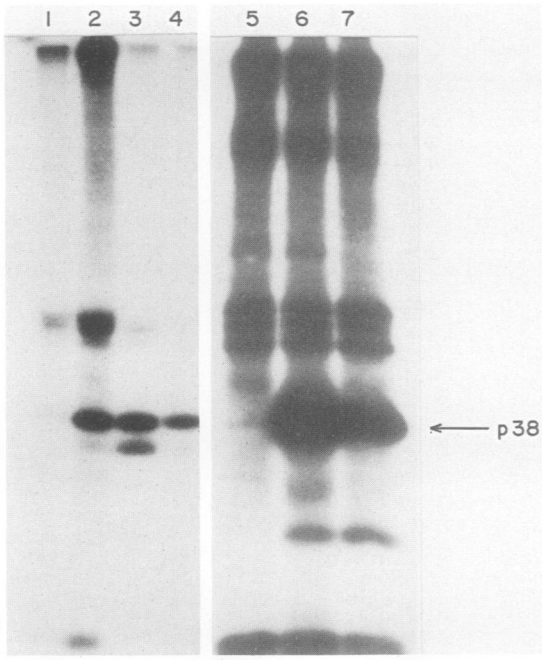

FIG. 6. Immunoprecipitation of phosphorylated p38 with antiserum against p38. The experiments were performed as described in the text. Lane 1, Preimmune serum; lane 2, 1:100 dilution of anti-p38 serum without normal rabbit serum in the first step; lane 3, 1:40 dilution of anti-p38 serum with normal rabbit serum in the first step; lane 4, 1:100 dilution of anti-p38 serum with normal rabbit serum in the first step. Lanes 5 to 7 show the starting material for immunoprecipitation, i.e., the in vitro phosphorylation products. Lane 5, Control; lanes 6 and 7, *ras* (50 μg/ml). Exposure time: lanes 1 to 4, 3 days at −70°C with intensifying screens; lanes 5 to 7, 12 h at −70°C with intensifying screens.

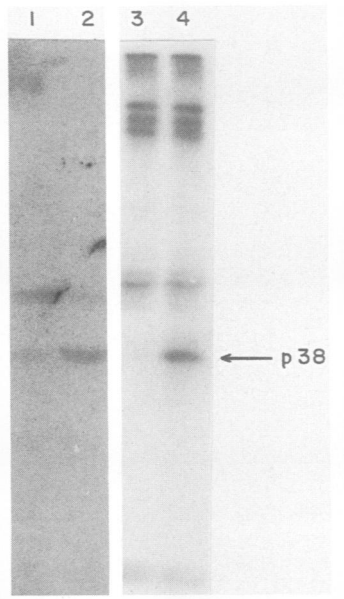

FIG. 7. In vivo stimulation of p38 phosphorylation by glucagon. Metabolic labeling with ^{32}P and immunoprecipitation with anti-p38 serum (1:40 dilution) were performed as described in the text. Lane 1, Control; lane 2, glucagon (20 μg/ml). Lanes 3 (control) and 4 (glucagon [20 μg/ml] plus 500 nM GTP) correspond to products of in vitro phosphorylation. Exposure time: lanes 1 and 2, 7 days at −70°C; lanes 3 and 4, 6 h at −70°C; both with intensifying screens.

could be reasonably certain that the antibodies raised were indeed against p38; they were monospecific and they recognized the native and phosphorylated forms of p38.

Stimulation of p38 phosphorylation by glucagon in vivo. To check whether glucagon stimulated p38-phosphorylation in vivo, we grew liver cells in phosphate-free Dulbecco modified Eagle medium in the presence of ^{32}P to label the intracellular nucleotides. At the end of the incubation, glucagon (20 μg/ml) was added, and after a further 5- min incubation, the cells were lysed and immunoprecipitated. Immunoprecipitations were done with validated serum against p38 (see above). p38 phosphorylation was stimulated at least two- to threefold by glucagon under these conditions (Fig. 7). To recognize the p38 band unambiguously, we loaded the products of in vitro phosphorylation reactions on the same gel along with the immunoprecipitated samples (Fig. 7, lanes 3 and 4).

To ascertain the nature of p38 phosphorylation which occurred in vivo, the gels after electrophoresis of immunoprecipitated samples were subjected to acid treatment before autoradiography. They p38 bands were excised from the acid-treated and untreated gels and counted for radioactivity. The phosphate moiety of p38 phosphorylated in vivo was acid labile just as for in vitro phosphorylation (Table 2).

Further, when the p38 bands phosphorylated by stimulation with glucagon in vitro and in vivo were subjected to a comparative one-dimensional peptide mapping, we observed that the phosphorylation occurred on the same site(s) (Fig. 8).

DISCUSSION

We established that glucagon stimulates the phosphorylation of p38 in vitro and in vivo. The phosphorylation in p38 in the presence of glucagon appeared identical with the phosphorylation taking place in the presence of *ras* proteins. Further, we showed that the phosphorylation of p38 stimulated by glucagon in vitro and in vivo occurs on the same site(s) and is of the same nature.

The enhancement of phosphorylation brought about by glucagon observed in vitro required the presence of guanine nucleotides. For reactions done with *ras* proteins, guanine nucleotides are already available in the bound form. The dependence of the phosphorylation reactions on guanine nucleotides suggests the involvement of G proteins in the phosphorylation of p38. In reaction pathways mediated by G proteins, the active form of the proteins is believed to be the GTP-bound molecule (17, 44). In the present experiments, glucagon stimulated the phosphorylation of p38 in the presence of both GTP and GDP. Since we used plasma membrane preparations for phosphorylation reactions, it is quite

TABLE 2. Acid lability of p38 phosphorylated in vivo

Description	^{32}P counts[a] (%)
Untreated	100
Acid treated	10
Background	9

[a] The gels were dried and autoradiographed with or without acid treatment. The p38 band in each case was excised from the dried gels, and Cerenkov counts were obtained.

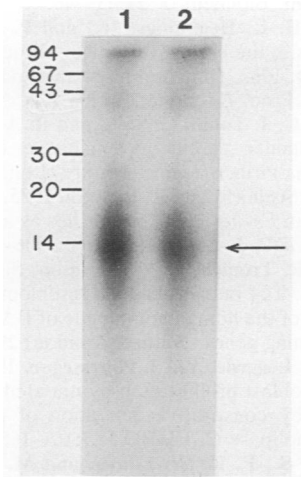

FIG. 8. Comparative peptide mapping of p38 phosphorylated in vitro (lane 1) and in vivo (lane 2). The experiment was performed as described in the text with 2 μg of V8 protease in each lane. Exposure time was 15 days at −70°C with intensifying screens. Molecular size standards (in kilodaltons) are shown on the left. The position of the phosphorylated peptide is indicated by an arrow.

possible that GTP was generated in the reactions from GDP by the dinucleotide kinase activity present in the preparations. This type of conversion of GDP to GTP will also explain the stimulatory activity of GDP-bound *ras* proteins. Observations similar to this have been made for yeast RAS2 proteins (11), the vertebrate adenylyl cyclase system (25), and G-protein-modulated mitogenesis with prolactin and interleukin-2 (46). Also, it has recently been demonstrated that glucagon regulates GDP-to-GTP conversion with subsequent activation of a G protein (23). We have also observed that GDPβS, an analog of GDP which cannot be converted to GTP, cannot stimulate p38 phosphorylation (unpublished observation). Another observation which would argue for the involvement of a G protein is the stimulation of p38 phosphorylation by AlF_4^-. As we showed (Table 1), AlF_4^- in the presence of GDP stimulates the phosphorylation significantly. Like G proteins, *ras* proteins also bind GTP and GDP and have GTPase activity (16, 31). Owing to these properties and their sequence homology with G proteins, *ras* proteins are considered to be similar to the former (2, 17). Hence, one would expect AlF_4^- to have the same effect on *ras* proteins also. In fact, AlF_4^- has been used to stimulate p21 *ras* in experiments designed to delineate its function (39). It has been shown with fluorescent GDP analogs that AlF_4^- induces conformational changes in the p21 *ras* molecule (39). Hence, a combination of *ras* and AlF_4^- would be equivalent to using the GTP-bound form of *ras*. We observed that the combination of *ras* with AlF_4^- stimulated p38 phosphorylation considerably (Table 1). The stimulation of any reaction by AlF_4^- has been accepted as one of the criteria for the involvement of G proteins (17).

Note that the acid-labile phosphorylation observed under the conditions of our experiments is novel. This is the first instance in which phosphorylation of a cellular protein has been identically stimulated by *ras* proteins and a peptide factor. At present, the exact nature of the linkage of the phosphate moiety is not known. Since it is labile in acid and also in strong, hot alkali, it is likely to be an acyl phosphate and probably not phosphohistidine as we thought earlier (20). The phosphorylation of p38 is likely to be physiologically significant, considering that the stimulation by glucagon occurs in vivo. We are examining the possible role of G proteins in p38 phosphorylation and that of phosphorylated p38 in signal transduction.

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