REGULATION OF A MICROSOMAL PROTEIN KINASE FROM MOUSE LIVER BY HEMIN AND THE INTERFERON INDUCER TILORONE

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SUMMARY

A 0.85 M KCl extract from mouse liver microsomes was shown to phosphorylate phosvitin and endogenous substrates. The corresponding protein-kinase(s) activity was inhibited *in vitro* by hemin (20-50 μM). Hemin blocked the phosphorylation of most of the phosphopolypeptides from this extract. The interferon inducer, tilorone, (which increased *in vivo* this protein-kinase(s) activity) prevented most of the inhibitory effect of hemin but had no effect in absence of hemin. The phosphorylation of the polypeptides of the extract was restored by tilorone, especially the component of MW 35 000.

INTRODUCTION

Recent data have indicated that the translational inhibitor, which blocks protein chain initiation in reticulocytes lysates deprived of hemin is a protein kinase independent of cyclic AMP (1). In this system, heme prevents the effect of the translational inhibitor. By contrast, dsRNA, an interferon inducer, inhibits protein synthesis (2) perhaps by activating the protein kinase which blocks the initiation step. Cells treated with interferon have also an altered capacity to translate mRNA into proteins (3,4). It has been shown that an inhibitor associated with ribosomes is responsible of this effect (4). *In vitro*, this inhibitory activity is strongly increased by dsRNA (5). A mechanism similar to that which exists in reticulocytes might explain the stimulation by dsRNA of the interferon-mediated inhibition of cell-free protein synthesis. Zilberstein *et al.* (6), Lebleu *et al.* (7) and Roberts *et al.* (8) have shown that phosphorylation of some proteins from cells treated by interferon is stimulated *in vitro* by dsRNA. These observations prompted us to investigate in mouse liver the *in vitro* effect of hemin and the *in vivo* effect of tilorone a strong interferon inducer (9), on the protein kinase activity associated with microsomes.

Footnote : protein kinase (E.C 2.7.1.37)
eucaryotic initiation factor 2 (IF-E2)

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MATERIAL AND METHODS

Hemin and phosvitin were obtained from Sigma Laboratories and (γ^{32}P)-ATP from the Radiochemical Center (Amersham, England). Tilorone, a product of Merrel Laboratories, was a generous gift from Dr I. Gresser. ATP was purchased from Boehringer-Mannheim, Germany.

Hemin was prepared as described by Woodward *et al.* (10).

Preparation of the microsomal protein kinase from mouse liver Male Swiss mice were sacrified by decapitation. The livers were quickly removed and homogenized in 250 mM sucrose, 1 mM $MgCl_2$, 50 mM Tris pH 7.5, (5 vol/g of liver). Nuclei, cells debris and mitochondria were pelleted by centrifugation at 15,000 g for 15 min and discarded. Microsomes were sedimented at 145,000 g_{max} for 90 min in a Spinco 50 Ti rotor. The microsomes were homogenized in 50 mM Tris pH 7.5, 250 mM sucrose, 1 mM $MgCl_2$ and 850 mM KCl (2 vol./g of liver) and sedimented at 110,000 g_{max} overnight. The supernatant contained the microsomal protein kinase used in the following experiments.

Determination of protein kinase activity In a final volume of 0.5 ml, the reaction mixture contained 50 mM Tris pH 7.5, 1 mM $MgCl_2$, 25 mM sucrose, 80 mM KCl, 0.75 mg of phosvitin when indicated, and 0.1 mM (γ^{32}P)-ATP. The reaction was initiated by adding ATP and was allowed to proceed for 12 min at 37°C. ^{32}P incorporation was determined as previously described (11). In some experiments, the histone kinase activity was measured. $MgCl_2$ was then increased to 8 mM, histones were substituted for phosvitin and 5 μM cyclic AMP was added.

Polyacrylamide gel electrophoresis in the presence of SDS Electrophoresis in the presence of SDS was performed according to the method of Laemmli (12) using a 15 % acrylamide separation gel and was carried out at 110 V for 5 h. After staining with Coomassie blue, gels were dehydrated. Autoradiography was performed by applying an X-ray film to the dry gel for 7-15 days.

RESULTS

I. Effect of hemin on microsomal protein kinase

In eukaryotic cells, protein kinases are associated with ribosomes and microsomes (13-16). Table 1 shows that the protein kinase of the mouse liver microsomal extract was more active on phosvitin than on histones under our test conditions. Most of the kinase activity is therefore of the phosvitin kinase type which is independent of cyclic AMP. We investigated the effect of hemin on the ^{32}P incorporation into phosvitin, histones and the endogenous substrates of the microsomal extract. Table 1 shows that hemin (50 μM) was a strong inhibitor of the microsomal protein kinase when tested on phosvitin or the endogenous substrates. However, no effect of hemin was observed when histones were used as the substrate. When tested with phosvitin, the ratio of the activity in the absence and the presence of

TABLE 1

EFFECT OF HEMIN ON MICROSOMAL PROTEIN KINASE

		cpm incorporated/mg protein substrate						
	% inhibition caused by 50 μM hemin	Phosvitin			Histones		Endogenous substrates (extract alone)	
		- hemin	+ hemin 50 μM	+ hemin 20 μM	- hemin	+ hemin 50 μM	- hemin	+ hemin 50 μM
ent I	75	81 000	20 000	-	-	-	-	-
ent II	72	106 500	30 440	-	-	-	39 400	6 630
ent III	88	68 300	12 000	-	19 800	16 960	-	-
ent IV	80	65 200	13 300	33 260	-	-	-	-

ein kinase activity was determined as indicated in Material and Methods. ific radioactivity of ATP was 1.5×10^5 cpm/nanomole. About 100 μg of protein-enzyme added to 0.5 ml of the reaction mixture. To determine incorporation into vitin and histones, cpm incorporated in endogenous substrates were substracted.

hemin was 75 to 87 % for 50 μM and 50 % for 20 μM hemin. When the endogenous substrates were tested, hemin also inhibited strongly the kinase (83 %).

II. Effect of tilorone on microsomal protein kinase

We investigated the effect of tilorone on the microsomal protein kinase. Tilorone hydrochloride, (2,7 bis 2-(diethyl amino) ethoxy fluoren-9-one dihydrochloride) is an active inducer of interferon (9). We observed only a slight inhibition of the protein kinase activity when tilorone was added at a concentration of 1 mM (Table 2). However when hemin was added with tilorone, we observed that the inhibitory effect of hemin was considerably reduced (Table 2). Tilorone restored 70-80 % of the activity measured in the absence of hemin. This effect of tilorone on the inhibitory activity of hemin was also observed when the concentration of tilorone was decreased to 0.1 mM.

III. Analysis by SDS electrophoresis of the phosphorylation products from the microsomal extract

The effect of hemin and tilorone on the phosphorylation

TABLE 2

IN VITRO EFFECT OF TILORONE ON THE MICROSOMAL PROTEIN KINASE

	cpm incorporated/mg substrate				
Compound(s) added	No addition	+ 50 μM hemin	+ tilorone 1 mM	+ hemin 50 μM + tilorone 1 mM	+ hemin 50 μM + tilorone 100 μM
Experiment I	61 400	9 000	-	47 600	43 200
Experiment II	104 000	30 000	91 200	73 700	-
Experiment III	58 300	13 320	-	-	52 000

Protein kinase activity was determined as indicated in Material and Methods with phosvitin as substrate. Specific radioactivity of ATP was $1.5\ 10^5$ cpm/nanomole. About 100 μg of protein-enzyme was added to 0.5 ml of the reaction mixture.

of the endogenous substrates was further analyzed by SDS electrophoresis. Fig. 1 (slot c) shows that there were several polypeptides phosphorylated in the absence of hemin. In the presence of hemin, the phosphorylation of most of the polypeptides was inhibited except for some components of high molecular weight (slot a). Tilorone in the presence of hemin (slot b) caused an increase in the phosphorylation of nearly all the components seen in the presence of hemin alone. In particular, tilorone caused the phosphorylation of a 35,000 molecular weight protein (see arrow in Fig 1) which was not easily seen in control extracts. Tilorone, in the absence of hemin, did not modify the pattern of phosphorylation (not shown).

IV. *In vivo* effect of tilorone on the microsomal protein kinase

Mice were injected intraperitoneally with tilorone (1.3 mg). Twenty hours later, the animals were sacrified and the microsomes isolated. Other mice were injected with physiological saline and treated in a similar manner. Finally, the microsomes were extracted with 0.85 M KCl as described above, and the protein kinase activity of these extracts was determined.

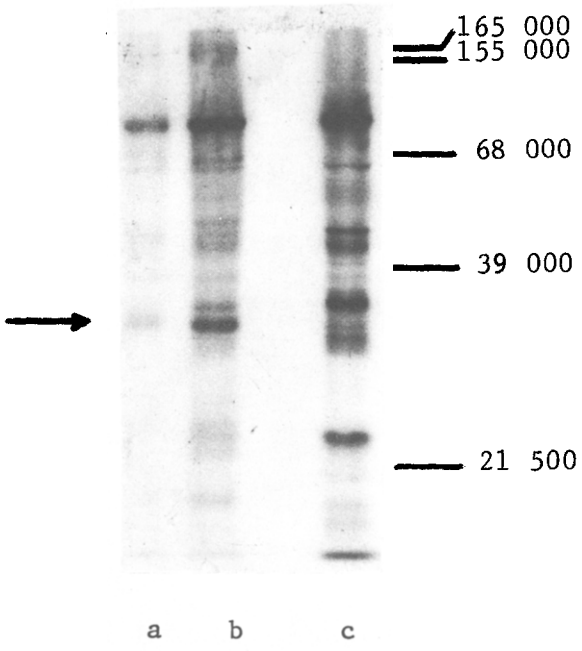

FIGURE 1

Effect of hemin and tilorone on the phosphorylation of the polypeptides from the 0.85 M KCl extract from mouse liver microsomes.

Microsomal extracts (1 mg protein/ml) were incubated with (γ^{32}P)-ATP and analyzed by SDS electrophoresis as described under Material and Methods.

a - in the presence of hemin
b - in the presence of hemin and tilorone
c - in the absence of hemin.

Arrow indicates the component of MW 35,000.
Molecular weight standard were trypsin inhibitor from soybean, 21,500 ; bovine serum albumin, 68,000 ; RNA polymerase from *E. coli* α subunit 39,000 ; β subunit, 155,000 ; β' subunit, 165,000.

When measured with phosvitin or endogenous substrates, the microsomal protein kinase activity was increased in animals treated with tilorone (Table 3).

DISCUSSION

The results presented here show that hemin, at a concentration of 50 µM, inhibits the cyclic AMP independent phosvitin kinase activity associated with mouse liver microsomes. A lesser inhibitory effect was also observed with 20 µM hemin. This effect of hemin was found when protein kinase activity was measured either with phosvitin or the endogenous substrates of the microsomal extract.

TABLE 3

IN VIVO EFFECT OF TILORONE ON MICROSOMAL PROTEIN KINASE

	cpm incorporated/mg protein substrate			
	incorporation into endogenous substrate (extract alone)		incorporation into phosvitin	
	+ tilorone	- tilorone	+ tilorone	- tilorone
Experiment I	64 800	18 400	84 800	43 700
Experiment II	67 200	26 100	69 500	42 000
Experiment III	-	-	66 600	44 000

Mice were sacrified 30 h after injection of tilorone. Specific radioactivity of ATP was 3×10^5 cpm/nanomole. About 40 µg of protein-enzyme was added to 0.5 ml of reaction mixture.

A moiety of these substrates probably correspond to proteins associated with polysomes which are phosphorylated *in vivo* (17).
No effect was observed when histones were used as the substrate.

Hemin (5-40 µM) stimulates protein synthesis in reticulocyte lysates (2) and other systems (2, 18) probably by acting on the initiation step. Several workers have shown that hemin prevents the effect of a translational inhibitor (2), which seems to be a cyclic AMP independent protein kinase phosphorylating the small subunit of the initiation factor IF-E2. Most authors (2, 19) think that, in reticulocytes lysates, hemin prevents the appearance of a new kinase, although Hirsch *et al.* (20) have found that hemin directly inhibits a protein kinase associated with ribosomes. In mouse liver, we found also that hemin inhibits a protein kinase present in microsomal extracts.

Tilorone, an interferon inducer, prevented most of the inhibitory effect of hemin and increased especially the phosphorylation of one component of molecular weight 35,000, this component might correspond to the small subunit of the initiation factor IF-E2. Tilorone might compete with hemin for a regulatory site of the kinase. Further work will be necessary to test this hypothesis.

When injected into mice, tilorone promoted an increase of the protein kinase activity of the microsomal extract. Since tilorone is not rapidly metabolized (21), this effect might be caused by the presence of tilorone in the microsomal extract. This hypothesis is unlikely because hemin also inhibits protein kinase activity in microsomal extracts from animals treated with tilorone (not shown). As tilorone induces interferon production, it is possible that the increase in protein kinase activity is due to the presence of interferon in animals. Several groups have shown that extracts of cells treated with interferon had a higher protein kinase activity as compared with control cells. This activity is specific for two or three polypeptides present in the cytosol or the KCl wash of the ribosomes (6). This protein kinase activity is increased by dsRNA which inhibits translation in extracts of L cells treated by interferon (5) and in reticulocyte lysates (2). It is interesting to note that interferon inducers with different chemical structures (dsRNA and tilorone) modulate the *in vitro* phosphorylation of proteins from the ribosomal wash, an effect which, in certain ways, is strikingly similar to the *in vivo* action of interferon (6).

Protein-kinase(s) can be an important factor for the control of protein synthesis in eukaryotic cells by phosphorylation of initiation factors (1,6-8, 19, 22).

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