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Biochemistry

Interferon, double-stranded RNA, and protein phosphorylation

[protein kinase/Ehrlich ascites tumor cell/poly(I)·poly(C)]

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ABSTRACT **We reported earlier that the addition of double-stranded RNA and ATP increases the endonuclease activity more in an extract of Ehrlich ascites tumor cells which have been treated with an interferon preparation than in a comparable extract from control cells. We report here that the addition of double-stranded RNA to an extract from Ehrlich ascites tumor cells which have been treated with an interferon preparation [or with the interferon inducer poly(I)·poly(C)] promotes the phosphorylation by [γ-^{32}P]ATP of at least two proteins: P_1 (molecular weight of 64,000) and P_2 (molecular weight of 37,000). Double-stranded RNA also promotes the phosphorylation of at least one (i.e., P_1) of these two proteins in an extract from cells which have not been treated with interferon, but the extent of phosphorylation is much smaller. Double-stranded RNA which has been degraded by RNase III, or DNA, does not promote the phosphorylation.**

Interferons are glycoproteins whose formation is induced in various animal cells upon infection by any of a large variety of viruses. They are released from the producing cells, are attached to other cells, and convert these into the "antiviral state." In interferon-treated cells which are in the antiviral state, the replication of a broad range of viruses is impaired (1). We have been investigating the molecular basis of this impairment. Many of our studies were performed with mouse Ehrlich ascites tumor (EAT) cells and reovirus (2, 3), a virus with a segmented double-stranded (ds) RNA genome (4).

The results of interferon treatment of EAT cells are manifested in cell extracts in various ways (5–10).

One result is that the rate of degradation of various exogenous mRNAs is faster in extracts from cells treated with interferon ($S30_{INT}$) than in extracts from control cells ($S30_C$), but only if the extracts are supplemented with ds RNA [e.g., from reovirus or poly(I)·poly(C)] and ATP (refs. 11 and 12; G. C. Sen, B. Lebleu, G. E. Brown, M. Kawakita, E. Slattery, and P. Lengyel, submitted for publication). We designated the enzyme(s) responsible for the faster RNA degradation in $S30_{INT}$ extracts as endonuclease$_{INT}$.

The activation of endonuclease$_{INT}$ is not impaired by inhibitors of protein synthesis. This makes it probable that the activation process does not involve *de novo* protein synthesis (12).

We considered that (*i*) ATP is required for the activation of endonuclease$_{INT}$ by ds RNA (12); (*ii*) some proteins are activated (or inactivated) by phosphorylation (13); (*iii*) the inhibition of peptide chain initiation in a heme-deficient reticulocyte lysate is due presumably to the inactivation of an initiation factor by phosphorylation (ref. 14; personal communication from P. Farrel *et al.* quoted in ref. 15), moreover there is a protein kinase associated with the inhibitor (16); and (*iv*) the inhibition of peptide chain initiation by ds RNA in lysates not deficient in heme might also be due to protein phosphorylation (15). We were prompted by these considerations to test the effect of ds RNA on protein phosphorylation in $S30_{INT}$ and $S30_C$.

The data presented in this communication reveal that ds RNA added to $S30_{INT}$ promotes the phosphorylation of at least two proteins by [γ-^{32}P]ATP. It promotes the phosphorylation of at least one of the same two proteins also in $S30_C$, but to a much smaller extent.

MATERIALS AND METHODS

Chemicals. [γ-^{32}P]ATP was obtained from New England Nuclear Corp., aurintricarboxylic acid from Eastman Organic Chemicals, Pronase from Calbiochem, proteinase K from Merck, DNase I (DPFF) and pancreatic RNase (RASE) from Worthington, poly(I) and poly(C) from Miles Laboratories, and DEAE-dextran from Pharmacia. Sparsomycin was a generous gift from the Upjohn Co., edeine from W. Szer, RNase III (prepared by R. Crouch) from J. Steitz, and λ and calf thymus DNA from P. Howard-Flanders.

Interferons. The specific activity of the partially purified preparation of mouse interferon was 1.1×10^6 vesicular stomatitis virus plaque reduction units (i.e., 1.1×10^7 units of the National Institutes of Health mouse reference standard) per mg of protein (17). The specific activity of the human fibroblast interferon was 10^4 units of National Institutes of Health reference standard for human interferon per mg of protein.

Double-Stranded Reovirus RNA (ds reo RNA). The preparation of ds reo RNA (free of adenylate-rich oligonucleotides) was based on published procedures (11). Where indicated, it was digested with RNase III to short fragments sedimenting slower than tRNA in sucrose gradients.

S30 Extracts. S30 extracts were prepared from control EAT cells and from EAT cells treated with 60 units of mouse interferon per ml (vesicular stomatitis virus plaque reduced in units) for 18 to 24 hr as described earlier (18) except that (*a*) the solution in which the cells were washed was supplemented with 12 mM glucose, (*b*) dithiothreitol was omitted from all solutions, and (*c*) aliquots of the S30s were frozen in liquid nitrogen right after centrifugation at $30,000 \times g$. $S30_{INT}$ and $S30_C$ extracts were used unless otherwise specified. By passing the $S30_C$ or $S30_{INT}$ extracts through a 30×2 cm Sephadex G-25 (medium) column which was equilibrated (and was eluted) with 20 mM Tris·Cl at pH 7.6, 80 mM KCl, 4 mM $MgCl_2$, and 6 mM 2-mercaptoethanol, the $S30_{INT.S}$ and $S30_{C.S}$ extracts were produced. S30 extracts from EAT cells treated with the interferon-inducer poly(I)·poly(C) (1) were prepared according to published procedures (11). The yield of vesicular stomatitis virus from infected cells in a single growth cycle was reduced by over 95% in cells treated with interferon, and by over 99% in those treated with poly(I)·poly(C). S30 extracts from EAT cells were

Abbreviations: ds RNA, double-stranded RNA; ds reo RNA, double-stranded reovirus RNA; EAT cells, mouse Ehrlich ascites tumor cells; $NaDodSO_4$, sodium dodecyl sulfate; $S30_{INT}$ and $S30_C$, extracts from cells treated with interferon and from control cells, respectively; $S30_{INT.S}$ and $S30_{C.S}$, extracts (from cells treated with interferon and from control cells, respectively) which have been passed through Sephadex-G-25; cAMP, adenosine 3′:5′-cyclic monophosphate.

used throughout unless otherwise indicated. S30 extracts (not preincubated and not Sephadex-treated) were prepared from HeLa S_3 cells either treated with 200 National Institutes of Health reference units of a human fibroblast interferon preparation per ml of cell culture fluid for 18 hr or from untreated HeLa cells according to Weber *et al.* (19). S30 extracts from HeLa cells were only used in the experiments shown in Fig. 2C.

Assay for Protein Phosphorylation in S30 Extracts. The reaction mixtures contained the following components in a final volume of 15 μl: 30 mM Tris·Cl at pH 7.6, 120 mM KCl, 6 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 10 μM[γ-^{32}P]ATP (about 2.5 μCi), and 0.3–0.5 A_{260} units of S30 extract of the type specified. The reaction was incubated at 30° for 3 to 5 min, and terminated by the addition of 10 μl of a solution containing 60 mM Tris·Cl at pH 7.6, 1% (wt/vol) sodium dodecyl sulfate (Na-DodSO$_4$), 1% (vol/vol) 2-mercaptoethanol, 15% (vol/vol) glycerol, and 0.05% (wt/vol) bromophenol blue. The samples were heated at 90° for 4 min. About 0.6–0.8% of the ^{32}P was transferred to hot trichloroacetic acid-insoluble material.

Aliquots of the heated samples (15 μl usually) were analyzed without further processing by electrophoresis on polyacrylamide gels and radioautography to determine the amount of ^{32}P transferred from [γ-^{32}P]ATP to individual protein bands. The electrophoresis system used was the discontinuous high-pH system described by Laemmli (20) except for the stacking gels, which contained 10% acrylamide. The slabs were 18 cm long by 1.6 mm thick. Electrophoresis was carried out at a current of 40 mA during migration through the stacking gel, 60 mA during the separation procedure, and was stopped when the tracking dye reached the bottom of the gel (ATP and phosphate were run off the gel). The gels were subjected to radioautography with Kodak NS54T x-ray film for 1–4 days, and the radioautographs were developed and scanned with a Joyce-Loebl densitometer. For molecular weight determinations (21), the gels were stained for proteins with 0.1% Coomassie brilliant blue prior to autoradiography.

RESULTS

Our preliminary experiments on protein phosphorylation were performed with $S30_C$ and $S30_{INT}$ extracts. We tested the transfer of ^{32}P from [γ-^{32}P]ATP into hot trichloroacetic acid-insoluble product from which the radioactivity could not be extracted with an alcohol:ether (50:50, vol/vol) mixture. We observed that ds RNA (1.5–5 μg/ml) increased protein phosphorylation in $S30_{INT}$ extracts by 20–30% without detectably affecting it in $S30_C$ extracts (data not shown).

Subsequently, to characterize the phosphorylated products, the incubated reaction mixtures were heated with NaDodSO$_4$ and fractionated by electrophoresis in NaDodSO$_4$ on polyacrylamide gels, and the position of labeled products in the gels was determined by radioautography. Fig. 1 shows that the addition of ds reo RNA (1.5 μg/ml) to a reaction mixture containing $S30_{INT}$ extract boosts the phosphorylation of one band (designated subsequently as P_1) to such a large extent that P_1 becomes the most heavily labeled band in the gel (compare slots 3 and 4 in Fig. 1). The addition of ds RNA also boosts the labeling of P_1 in $S30_C$ extracts, but to a much lesser extent than in $S30_{INT}$ extracts (compare slots 2 and 1, and 4 and 3, in Fig. 1).

These observations have been repeated with the same qualitative results with all the seven different $S30_{INT}$-$S30_C$ extract pairs tested.

The difference in the extent of the ds RNA-promoted phosphorylation between $S30_{INT}$ and $S30_C$ extracts might be due

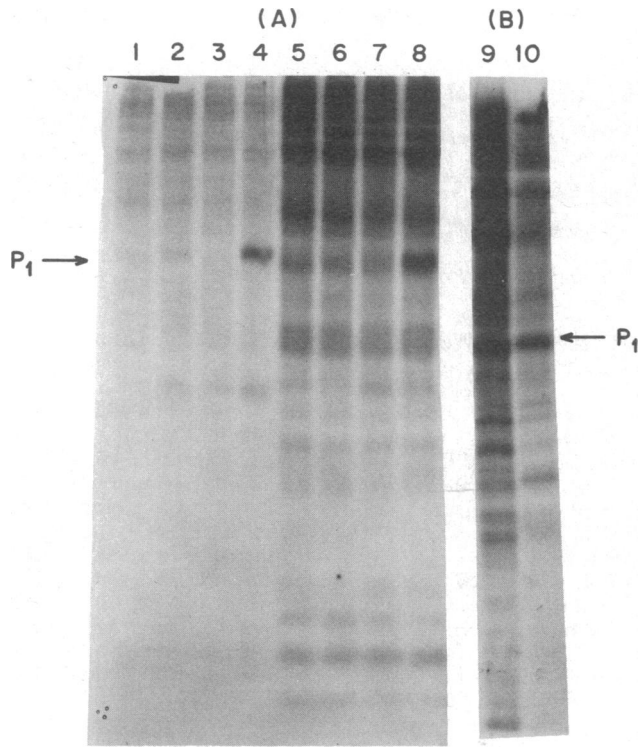

FIG. 1. The extent of phosphorylation of band P_1 as promoted by ds RNA is larger in $S30_{INT}$ than in $S30_C$ extracts. (A) Radioautographs of protein phosphorylation with and without ds reo RNA in Sephadex-treated and untreated extracts from interferon-treated and control cells. The reaction mixtures included (*1*) $S30_C$; (*2*) $S30_C$ + ds RNA; (*3*) $S30_{INT}$; (*4*) $S30_{INT}$ + ds RNA; (*5*) $S30_{C.S}$; (*6*) $S30_{C.S}$ + ds RNA; (*7*) $S30_{INT.S}$; (*8*) $S30_{INT.S}$ + ds RNA. (B) Radioautographs of protein phosphorylation with ds reo RNA in Sephadex-treated extracts from interferon-treated cells at low and high ATP concentrations. The reaction mixtures included (*9*) $S30_{INT.S}$ + ds RNA (10 μM ATP); (*10*) $S30_{INT.S}$ + ds RNA (120 μM ATP). Each reaction mixture was supplemented with 10 μM[γ-^{32}P]ATP and reaction mixture (*10*) was also supplemented with 110 μM of unlabeled ATP. Where indicated, 1.5 μg/ml of ds RNA from reovirus was added. (A) and (B) are sets of radioautographs from polyacrylamide gel electrophoresis of two separate experiments. P_1 is a protein band whose phosphorylation is promoted by ds RNA.

in principle to a difference in the concentration of small molecules [e.g., ATP, adenosine 3′:5′-cyclic monophosphate (cAMP)] present in the two types of extracts. To check this possibility, we repeated the experiments with $S30_{INT.S}$ and $S30_{C.S}$ (i.e., extracts from cells treated with interferon and from control cells which had been passed through Sephadex G-25 to remove small molecules). Fig. 1 (slots 5–8) reveals that small molecules may not account for the difference: ds RNA boosts the phosphorylation of P_1 in $S30_{INT.S}$ extracts greatly and in $S30_{C.S}$ ones only slightly if at all.

The labeling of proteins is much more pronounced in the extract treated with Sephadex than in the untreated extract. This is as expected, because the added labeled ATP is diluted by endogenous ATP only in $S30_{INT}$ and $S30_C$ extracts but not in $S30_{INT.S}$ and $S30_{C.S}$ ones.

It is remarkable that where ds RNA is added to $S30_{INT}$ extracts, P_1 contains the most isotope whereas in $S30_{INT.S}$ extracts, with the same conditions, there are other bands that either contain more or at least about the same amount of isotope as P_1. It should be noted that the incorporation of isotope into these other bands is not affected by ds RNA. The total concentration of ATP is lower in $S30_{INT.S}$ (which is free of endogenous ATP)

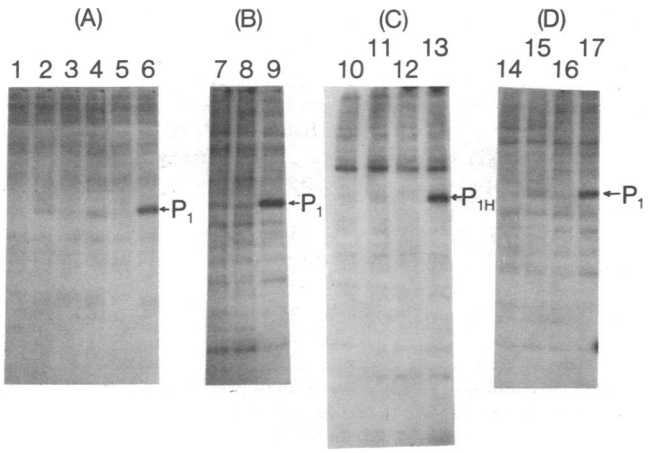

FIG. 2. The extent of phosphorylation of band P_1 as promoted by ds reo RNA is larger in extracts from cells in the antiviral state than in extracts from control cells. Effects on the extent of the phosphorylation of band P_1 in the extract from mouse EAT cells of (A) the length of treatment of cells with a mouse interferon preparation, and (B) the treatment of cells with a homologous (mouse) and heterologous (human) interferon preparation. (C) The effect on the extent of the phosphorylation of band P_{1H} in the extract of human HeLa cells of the addition of ds reo RNA and of the treatment of the cells with a human interferon preparation. (D) Effect on the extent of the phosphorylation of band P_1 in the extract of mouse EAT cells of ds reo RNA and of the exposure of cells to the interferon inducer poly(I)·poly(C). The extracts added to the reaction mixtures in A were from control EAT cells (*1, 2*), EAT cells treated with mouse interferon for 1 hr (*3, 4*) or from EAT cells treated for 24 hr (*5, 6*). The reaction mixtures were incubated without (*1, 3, 5*) or with 1.5 μg/ml of ds reo RNA (*2, 4, 6*). The extracts added to the reaction mixture in (B) were from control EAT cells (*7*), EAT cells treated with 200 units/ml of human fibroblast interferon for 18 hr (*8*), or EAT cells treated with 60 units/ml of mouse interferon for 18 hr (*9*). The reaction mixtures were incubated with 1.5 μg/ml of ds reo RNA. The extracts added to the reaction mixture in (C) were from control HeLa cells (*10, 11*) or HeLa cells treated with 200 units/ml of human fibroblast interferon for 18 hr (*12, 13*). The reaction mixtures were incubated without (*10, 12*) or with 1.5 μg/ml of ds reo RNA (*11, 13*). The extracts added to the reaction mixtures in (D) were from cells treated with DEAE-dextran (*14, 15*) or cells treated with DEAE-dextran and the interferon inducer poly(I)·poly(C) (*16, 17*). The reaction mixtures were incubated without (*14, 16*) or with 1.5 μg/ml of ds reo RNA (*15, 17*). [DEAE-dextran added together with poly(I)·poly(C) potentiates the effect of the polynucleotide in inducing interferon, but DEAE-dextran alone is not known to induce interferon.] (A), (B), (C), and (D) are sets of radioautographs from polyacrylamide gels of four separate experiments.

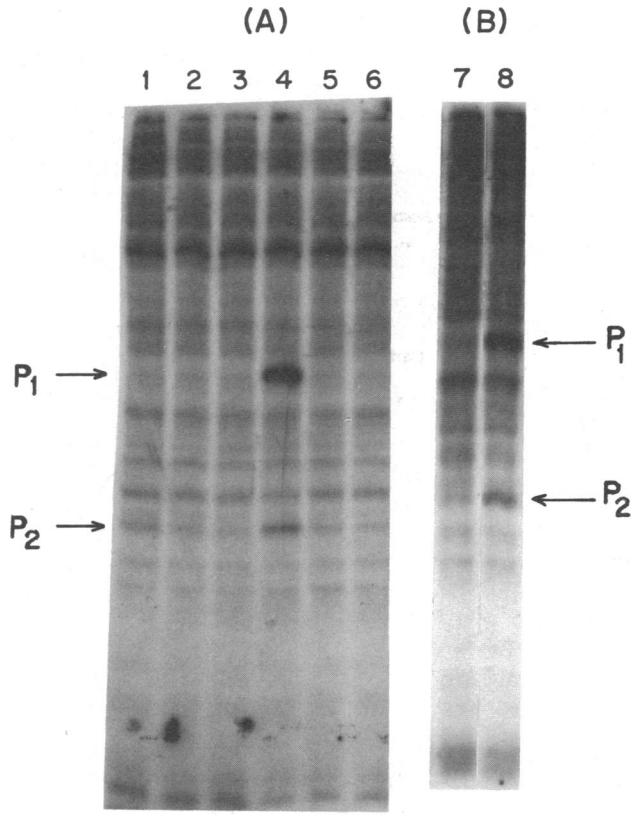

FIG. 3. Protein phosphorylation in $S30_{INT}$ extracts is promoted by ds reo RNA or by poly(I)·poly(C) but not by DNA or ds reo RNA which has been degraded to short fragments. $S30_{INT}$ extract was incubated in reaction mixtures without ds RNA (*1*) and (*7*), with 1.5 μg/ml of λ bacteriophage DNA (*2*), 1.5 μg/ml of calf thymus DNA (*3*), 1.5 μg/ml of ds reo RNA (*4*), 1.5 μg/ml of ds reo RNA which had been degraded by treatment with RNase III (*5*), 1.5 μg/ml of ds reo RNA which had been degraded by treatment with pancreatic RNase and RNase III (*6*), or 1.5 μg/ml of poly(I)·poly(C) (*8*). (A) and (B) are sets of radioautographs from polyacrylamide gels from two separate experiments. P_1 and P_2 are protein bands whose phosphorylation is promoted by ds RNA.

than in $S30_{INT}$ extracts (which is not). Consequently, the apparent discrepancy in the incorporation pattern between the $S30_{INT}$ and $S30_{INT.S}$ extracts might be accounted for by assuming that the kinase which phosphorylates P_1 has a lower affinity for ATP than the other kinases which phosphorylate other bands. To test this assumption, we increased the unlabeled ATP concentration in the reaction mixture containing $S30_{INT.S}$ extract from 10 μM (the concentration used in slot 9) to 120 μM (slot 10). This results, as expected, in a decrease in the ^{32}P-labeling of all bands, but under these conditions P_1 becomes the (or at least one of the) most extensively ^{32}P-labeled bands in $S30_{INT.S}$ extracts too. Thus, the results are in accord with the assumption.

Correlation Between the Establishment of an Antiviral State in Cells Treated with Either Homologous Interferon or the Interferon Inducer Poly(I)·Poly(C), and the Changes in the ds RNA-Promoted Protein Phosphorylation in Their Extracts. Because our interferon is not pure, we cannot prove that the above changes in the phosphorylation pattern have been induced by interferon and not by other components in the interferon preparation.

The following results are consistent with the possibility that the changes are induced by interferon: (*a*) the increase in the ds RNA-promoted P_1 phosphorylation is much more pronounced in an extract from cells which were treated with interferon for 24 hr than in one from cells treated for 1 hr (Fig. 2A). At the same time, it is known that the induction of the state in which virus replication is inhibited the most requires an exposure of cells to interferon for several hours (22). (*b*) Treatment of the mouse EAT cells with heterologous (i.e., human fibroblast) interferon has no effect on the ds RNA-promoted P_1 phosphorylation in the cell extract (Fig. 2B). However, the treatment of human HeLa cells with the same preparation of human fibroblasts interferon increases the ds RNA-promoted phosphorylation in the HeLa extract of the band designated as P_{1H} (Fig. 2C). (*c*) The ds RNA-promoted phosphorylation of P_1 is also increased in an extract from EAT cells which have not been treated with interferon, but with the interferon inducer poly(I)·poly(C) (Fig. 2D).

DNA or Degraded ds RNA Do Not Substitute for ds RNA in Promoting Protein Phosphorylation in $S30_{INT}$. The radioautographs in Fig. 3 reveal that λ DNA (1.5 μg/ml; slot 2)

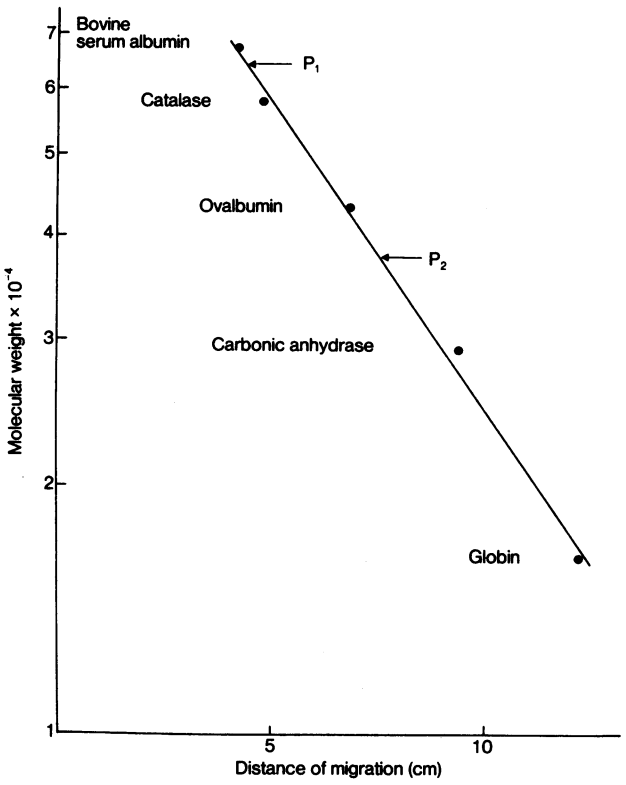

FIG. 4. Determination of the molecular weights of the phosphorylated proteins P_1 and P_2 by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$. $S30_{INT}$ extract was incubated with [γ-^{32}P]ATP and ds RNA. The following proteins were used as molecular weight markers: rabbit globin (16,000), carbonic anhydrase (24,000), ovalbumin (43,000), catalase (57,000), and bovine serum albumin (67,000). The position of the marker proteins in the gels was determined by staining.

or calf thymus DNA (1.5 μg/ml; slot 3) or ds reo RNA which has been exhaustively digested with RNase III alone (slot 5, refs. 23 and 24) or with RNase III and pancreatic RNase (slot 6) do not promote protein phosphorylation in $S30_{INT}$ extracts whereas, as also shown earlier, ds reo RNA does (cf. slot 4 with slot 1). The ds polyribonucleotide poly(I)·poly(C) (1.5 μg/ml; cf. slot 8 with slot 7) can replace ds reo RNA in promoting protein phosphorylation. Thus, ds RNA does not have to be of viral origin to exert this activity.

The results in Fig. 3 also indicate that ds reo RNA and ATP promote the phosphorylation of both P_1 and a second band designated as P_2 (cf. slot 4 with slot 1). P_2 is, however, less phosphorylated than P_1 and therefore cannot be seen clearly in Figs. 1 and 2. The promotion of phosphorylation of P_2 by ds RNA and ATP could be detected so far in five out of seven EAT $S30_{INT}$ preparations tested.

Dependence of the Extent of P_1 Phosphorylation in $S30_C$ and $S30_{INT}$ Extracts on the Concentration of ds reo RNA in the Reaction Mixture. The data in Table 1 reveal that 0.06 μg/ml of ds reo RNA causes an appreciable increase in P_1 phosphorylation and 0.3 μg/ml causes maximal phosphorylation. Addition of 1.5 or 5 μg/ml of ds reo RNA affects phosphorylation similarly to the addition of 0.3 μg/ml of ds reo RNA. It should be noted that the extent of P_1 phosphorylation without ds RNA (not shown) was indistinguishable from that in the presence of 0.0015 μg/ml of ds reo RNA.

The data in Table 1 also indicate that (as already shown in Figs. 1 and 2) ds reo RNA (1.5 μg/ml) promotes phosphorylation of P_1 in $S30_C$ extracts too, but clearly to a much lesser extent than in $S30_{INT}$ extracts.

Table 1. Effects of the concentration of ds reo RNA on the phosphorylation of protein P_1 by [γ-^{32}P]ATP in $S30_C$ and $S30_{INT}$ extracts

ds reo RNA (μg/ml)	Labeling of P_1 in cell extracts (in arbitrary units) $S30_C$	$S30_{INT}$
0.0015	3	12
0.06	—	27
0.3	—	65
1.5	7	51
5.0	—	70

$S30_C$ and $S30_{INT}$ extracts were incubated with [γ-^{32}P]ATP(10 μM) and ds reo RNA at the concentrations indicated. The incubated reaction mixtures were fractionated by electrophoresis in $NaDodSO_4$ on polyacrylamide gels. The radioautographs of the gels were scanned with a Joyce-Loebl microdensitometer. The amount of label in band P_1 was determined from the scans by planimetry. To take into account the variation in the amount of radioactivity applied to the gels from the various reaction mixtures, the optical density of a particular P_1 band was normalized against the total optical density in all other bands originating from the same reaction mixture.

Characterization of Phosphorylated P_1 and P_2 As Phosphoproteins. P_1 and P_2 were phosphorylated for these experiments by incubating $S30_{INT}$ extracts with[γ-^{32}P]ATP and 1.5 μg/ml of ds reo RNA for 5 min. The extent of radioactive labeling in P_1 and P_2 (as revealed in radioautographs of the gel from electrophoresis of the reaction mixtures) was not decreased by further incubation of the reaction mixtures at 30° for 20 min with either pancreatic RNase (0.1 mg/ml) or DNase (0.5 mg/ml). However, further incubation in the above conditions with proteolytic enzymes (i.e., Pronase, 0.5 mg/ml or proteinase K, 0.5 mg/ml) resulted in the disappearance of label from P_1 and P_2 (as well as from the other radioactively labeled bands). Repeated extraction of the reaction mixtures with a lipid solvent (i.e., ether) did not affect the amount of label in P_1 and P_2 (data not shown).

These results are consistent with the possibility that phosphorylated P_1 and P_2 may be phosphoproteins.

Determination of the Molecular Weights of P_1 and P_2. The molecular weight of phosphorylated P_1 and P_2 was determined by comparing their distance of migration by electrophoresis on polyacrylamide gels in $NaDodSO_4$ with those of marker proteins of known molecular weight (Fig. 4) (21). The molecular weight of P_1 is about 64,000, that of P_2 about 37,000.

DISCUSSION

Our results indicate that: (*i*) ds RNA and ATP increase both $endonuclease_{INT}$ activity (12) and the phosphorylation of proteins P_1 and P_2 more in $S30_{INT}$ than in $S30_C$ extracts. (*ii*) Degraded ds RNA or DNA do not substitute for ds RNA. (*iii*) The ds RNA and ATP promoted activation of $endonuclease_{INT}$ is not impaired by the inhibition of protein synthesis (12). Furthermore, recent experiments on the ds RNA and ATP promoted phosphorylation in $S30_{INT}$ extracts in the presence of inhibitors of peptide chain initiation (i.e., aurintricarboxylic acid or edeine), peptide chain elongation (i.e., sparsomycin) (25) or agents inhibiting protein synthesis by cleaving mRNA and tRNA (pancreatic RNase) revealed that the phosphorylation process is also independent of protein synthesis (data not shown).

The apparent similarity in the above characteristics between endonuclease$_{INT}$ activation and P_1 and P_2 phosphorylation is an inducement for the further comparison of the two processes. Especially one would like to know the relationship between P_1, P_2, and endonuclease$_{INT}$. The data available indicate that both ds RNA and ATP can be destroyed after they have activated endonuclease$_{INT}$ without causing a rapid cessation of endonuclease$_{INT}$ activity (G. C. Sen, B. Lebleu, G. E. Brown, M. Kawakita, E. Slattery, & P. Lengyel, in preparation). These findings suggest that protein phosphorylation may serve either to activate the enzyme or to inactivate an inhibitor of the enzyme.

The proposal that the inhibition of peptide chain initiation by ds RNA, in reticulocyte lysates, might be due to inactivation of an initiation factor by phosphorylation (14), on the one hand, and the fact that the translation of mRNA is more prone to inhibition by ds RNA in S30$_{INT}$ than in S30$_C$ extracts from L cells (26), on the other hand, make it conceivable that P_1 or P_2 phosphorylation might be related to the inactivation of the initiation factor in question.

Because the activity of many protein kinases is controlled by cAMP (27), we were prompted to test the effect of cAMP on P_1 and P_2 phosphorylation in S30$_{INT}$. We find that cAMP between 0.1 μM to 0.1 mM does not substitute for ds RNA and, in the presence of ds RNA, does not noticeably affect the extent of phosphorylation. At higher concentrations (1 mM), either cAMP or GTP (5 mM) impairs protein phosphorylation (data not shown).

At least *in vitro*, the effects of ds RNA and ATP on protein phosphorylation are not restricted to EAT cell extracts. The addition of ds RNA and ATP *also* promotes the phosphorylation of at least one protein in extracts from HeLa cells treated with a partially purified preparation of human fibroblast interferon, and to a much lesser extent in extracts from untreated HeLa cells (Fig 2C; S. Shaila and B. Lebleu, in preparation).

All our experiments on protein phosphorylation and endonuclease$_{INT}$ activation have been performed so far with crude extracts. The elucidation of the molecular basis for the promotion of protein phosphorylation by ds RNA obviously requires the fractionation of the system. Finally, experiments with intact cells are needed (*i*) to determine whether or not the results obtained with cell extracts reflect faithfully upon the processes taking place in intact cells, (*ii*) to establish the relationship (if any) between protein phosphorylation and endonuclease$_{INT}$ activation, and (*iii*) to determine the relevance (if any) to the functioning of the interferon system.

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