Vol. 101, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 30, 1981 Pages 680-688

MECHANISM OF INTERFERON ACTION
Inhibition of pppA(2'p5'A)$_n$-dependent Ribonuclease Activity in Micrococcal Nuclease-treated Mouse L Cell-free Extracts

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Received June 23, 1981

SUMMARY. The ability of nonpreincubated as compared to micrococcal nuclease-treated mouse L cell-free extracts supplemented with 2'-deoxythymidine-3',5'-diphosphate (pTp) and ethyleneglycol-bis (β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) to catalyze the 2',5'-oligoadenylate-dependent degradation of reovirus [^{3}H]mRNA was investigated. 2',5'-Oligo A tetramer enhanced degradation in nonpreincubated but not micrococcal nuclease-treated extracts. Neither pTp nor EGTA significantly affected the 2',5'-oligo A-dependent degradation in nonpreincubated extracts. The presence of both micrococcal nuclease and calcium was required to establish the subsequent reduction in both 2',5'-oligo A-dependent and -independent degradation observed in micrococcal nuclease-treated extracts containing pTp and EGTA.

INTRODUCTION

Two enzymic activities, a protein kinase and a 2',5'-oligoadenylate synthetase, are induced by most if not all interferon (IFN) species in a wide variety of IFN sensitive animal cells (1,2). The protein kinase catalyzes the phosphorylation of a ribosome-associated protein, designated P_1, and the small (α) subunit of protein synthesis initiation factor eIF-2 (3). The 2',5'-oligoadenylate (2',5'-oligo A) synthetase catalyzes the synthesis of a family of oligonucleotides of the structure pppA(2'p5'A)$_n$ (4) which activate an endoribonuclease that cleaves on the 3'-side of UN sequences of both viral and cellular RNAs (5,6). Both the kinase and the 2',5'-oligo A synthetase are dependent upon the presence of RNA with double-stranded character for activation following their induction by IFN.

0006-291X/81/140680-09$01.00/0

Recent studies indicate that changes in protein phosphorylation may play a primary role in regulating the translation of viral mRNAs in IFN-treated mouse cells. The 2',5'-oligo A synthetase is induced by IFN in mouse embryonal carcinoma (EC) cells to levels comparable to those found in differentiated cells; however, IFN does not induce either the protein kinase or an antiviral state as measured with wild-type vesicular stomatitis virus in EC cells (7,8). Conversely, IFN-mediated inhibition of viral protein synthesis may occur in the absence of detectable mRNA degradation but in the presence of protein phosphorylation (9). Mouse L cell-free protein synthesizing systems prepared from IFN-treated cells and made dependent upon exogenously added reovirus mRNA by treatment with micrococcal nuclease (10,11) catalyze the phosphorylation of P and eIF-2α in the presence of dsRNA but do not catalyze IFN-enhanced degradation of reovirus [^{3}H]mRNA in the presence of either dsRNA or 2',5'-oligo A (9).

This communication reports our results concerning the molecular basis by which the pppA(2'p5'A)$_n$-dependent ribonuclease activity is blocked in micrococcal nuclease-treated as compared to nonpreincubated cell-free extracts prepared from interferon-treated mouse L cells. The results indicate that digestion of endogenous cellular RNA by the micrococcal nuclease during the preparation of the mRNA-dependent protein synthesizing system is required to establish the apparent inhibition of the 2',5'-oligo A-dependent ribonuclease. Neither pTp nor EGTA directly affect the activity of either the 2',5'-oligo A synthetase or the 2',5'-oligo A-dependent ribonuclease in mouse L cells.

MATERIALS AND METHODS

Materials. Micrococcal nuclease purified from the Foggi strain of *Staphylococcus aureus* was from Sigma. 2'-Deoxythymidine-3',5'-diphosphate (pTp) was from either Calbiochem or P.L. Biochemicals; uridine-3',5'-diphosphate (pUp) was from P.L. Biochemicals. The tetramer of 2',5'-oligoisoadenylate, pppA(2'p5'A)$_3$ (2',5'-oligo A), was ^{3}H-labeled (1 Ci/mmole) and was generously provided by Dr. D.A. Eppstein, Syntex Research, Palo Alto, California. All other materials were as previously described (9).

Cell-free Extracts. Nonpreincubated and micrococcal nuclease-treated cell-free S-10 extracts were prepared as recently described (9) from mouse L fibroblast cells grown in suspension culture at 37° in Joklik's modification of Eagle's MEM (GIBCO) containing 1.4% fetal and 5.6% newborn calf serum (FLOW).

Extracts from IFN-treated L cells were prepared from cultures treated for 20 hr with 100 units/ml of Newcastle disease virus-induced mouse L_{929} cell IFN (12). The protein concentration of the extracts ranged from 10 to 12 mg/ml as determined by the phenol reagent method of Lowry (13).

RNAs. Reovirus double-stranded genome RNA (dsRNA) was isolated from purified virions (14). Reovirus [^{3}H]mRNA (2400 cpm/μg) was prepared as the *in vitro* transcription product (15) in the presence of 10μM S-adenosyl-L-methionine and 25 μCi/ml [^{3}H]ATP, and was purified as described (12).

RNA Degradation Assays. Degradation of reovirus [^{3}H]mRNA was routinely assayed by measuring the formation of cold perchloric acid-uranyl acetate soluble ^{3}H-oligonucleotides (16). The standard reaction mixture (25 μl) was similar to that utilized for measuring cell-free protein synthesis as described previously (9), except that the creatine phosphokinase was reduced to 10 μg/ml. Reovirus dsRNA (1 μg/ml) was added as indicated. 2',5'-oligo A tetramer, pTp, pUp, and EGTA were added at the concentrations specified in each experiment. Incubations were at 30° for the amount of time indicated.

The degradation of reovirus [^{3}H]mRNA was also determined by sucrose density gradient centrifugation using 15-30% linear gradients as previously described (17). Samples were incubated for 30 min at 30° in a reaction mixture as described above, except that the final volume was 50 μl and contained 6 μg reovirus [^{3}H]mRNA. 2',5'-Oligo A tetramer (3×10^{-8} M unless otherwise specified) and dsRNA (1μg/ml) were added as indicated. Centrifugation was for 16 hr at 29,000 rpm in a SW41 rotor at 25°. Fractions (0.4 ml) were collected with an ISCO model D fractionator, and the radioactivity per fraction was measured in Aquasol II cocktail (New England Nuclear) with a Beckman model LS-230 liquid scintillation system.

RESULTS AND DISCUSSION

The degradation of reovirus [^{3}H]mRNA catalyzed by cell-free extracts prepared from IFN-treated cells is enhanced by the addition of either dsRNA or 2',5'-oligo A (9, 16). The enhanced degradation is due to the activation of a latent, constitutive endoribonuclease by the family of related 2',5'-oligo A oligomers which are synthesized from ATP in the presence of dsRNA by an IFN-induced oligoadenylate synthetase (4,16). The ability of the purified 2',5'-oligo A tetramer, pppA(2'p5'A)$_3$, to activate the 2',5'-oligo A-dependent RNase present in extracts prepared from IFN-treated mouse L cells is shown in Figure 1. The degradation of reovirus [^{3}H]mRNA was quantitated by sucrose density gradient centrifugation (17). With nonpreincubated extracts prepared from IFN-treated cells (Fig. 1A), the extent of enhancement of [^{3}H]mRNA degradation depended upon the concentration of 2',5'-oligo A tetramer added to the reaction mixture. A 50% activation of the 2',5'-oligo A-dependent nuclease was obtained at a final tetramer concentration between 3 and 10 nanomolar;

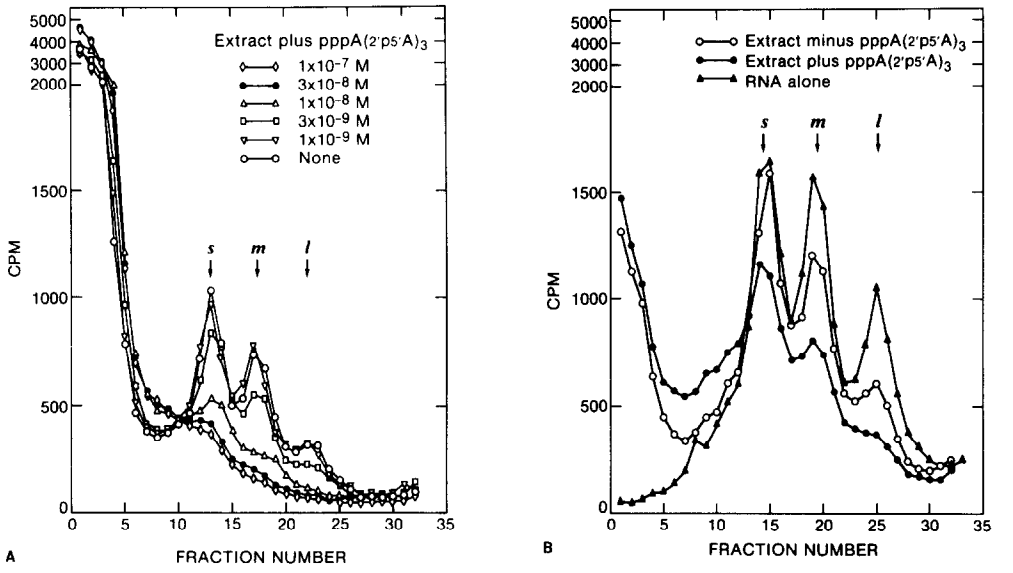

Figure 1. Effect of pppA(2'p5'A)$_3$ on the degradation of reovirus [^{3}H]mRNA by (A) nonpreincubated and (B) micrococcal nuclease-treated S-10 extracts prepared from IFN-treated cells. The concentration of tetramer used in B was 3 X 10^{-8} M. Analysis was by sucrose density gradient centrifugation as described under "Materials and Methods".

3 X 10^{-8} M was a saturating tetramer concentration for nuclease activation in nonpreincubated S-10 extracts (Fig. 1A). In contrast to the results obtained with nonpreincubated S-10 extracts, 2',5'-oligo A tetramer at a final concentration of 3 X 10^{-8} M did not significantly enhance the degradation of reovirus [^{3}H]mRNA catalyzed by micrococcal nuclease-treated extracts (Fig. 1B). Furthermore, the extent of degradation of reovirus mRNA in the absence of added 2',5'-oligo A tetramer was also greatly reduced in micrococcal nuclease-treated extracts (Fig. 1B) as compared to nonpreincubated extracts not treated with micrococcal nuclease (Fig. 1A). The results shown in Figure 1 obtained with purified 2',5'-oligo A tetramer are consistent with results recently reported (9) obtained with a mixture of 2',5'-oligo A oligomers.

EGTA (10) and pTp (11) are routinely added to final concentrations of 2 mM and 0.12 mM, respectively, to inhibit the micrococcal nuclease used to prepare cell-free protein synthesizing systems dependent upon exogenously added mRNA (9). In order to determine whether the differential effect of

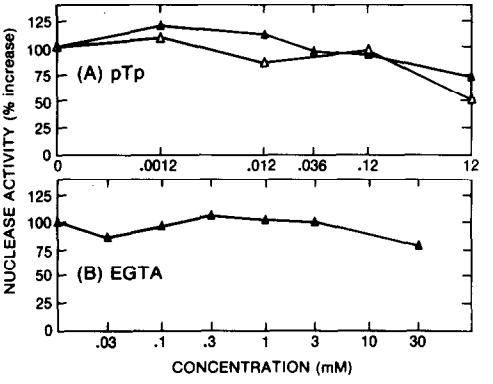

Figure 2. Effect of (A) pTp and (B) EGTA on 2',5'-oligo A-dependent degradation of reovirus [^{3}H]mRNA in the presence of either 3 X 10^{-8} M pppA(2'p5'A)$_3$ (▲——▲) or 1 µg/ml reovirus dsRNA (Δ——Δ). Analysis was by formation of cold acid-soluble ^{3}H-oligonucleotides as described under "Materials and Methods". The % increase in nuclease activity was calculated as follows: [(activity plus 2',5'A - activity minus 2',5'A) in the presence of pTp or EGTA divided by (activity plus 2',5'A - activity minus 2',5'A) in the absence of pTp and EGTA] X 100. Incubation was for 30 min at 30°.

2',5'-oligo A on nuclease activity in micrococcal nuclease-treated as compared to nonpreincubated extracts (Fig. 1; ref. 9) was due to the presence of EGTA and/or pTp, the following experiments were performed. The effect of pTp, a very potent inhibitor of micrococcal nuclease (18), on 2',5'-oligo A-dependent nuclease activity in nonpreincubated S-10 extracts was examined (Figure 2). pTp concentrations from 1.2 X 10^{-6} M to 1.2 X 10^{-3} M did not significantly affect the degradation of reovirus [^{3}H]mRNA by the 2',5'-oligo A-dependent nuclease activated by 3 X 10^{-8} M 2',5'-oligo A tetramer, as quantitated by formation of acid-soluble ^{3}H-oligonucleotides (Fig. 2A) or by sucrose density gradient centrifugation (data not shown). An analogue of pTp, pUp, also did not affect the activity of the 2',5'-oligo A-dependent nuclease (data not shown). In addition, the divalent cation chelating agent, EGTA, at final concentrations from 3 X 10^{-5} M to 3 X 10^{-2} M did not inhibit the 2,5'-oligo A-dependent nuclease activated by 3 X 10^{-8} M tetramer in nonpreincubated extracts (Fig. 2B).

The possibility was considered that the reduction in 2',5'-oligo A-dependent degradation observed with micrococcal nuclease-treated extracts may be due to

Table I

Degradation of Reovirus [^{3}H]mRNA by Extracts from IFN-treated Mouse L Cells.*

Experiment	[^{3}H]mRNA Degraded (a) *minus* 2',5'-oligo A	(b) *plus* 2',5'-oligo A	Enhancement by 2',5'-oligo A
	cpm	*cpm*	*%*
A. 10 min on ice with:			
no addition	2773	4272	≡ 100
EGTA, pTp	2371	3397	68
MN, Ca^{++}; then EGTA, pTp after 10 min	2398	3367	65
10 min at 25°C with:			
no addition	2839	4135	86
EGTA, pTp	2311	3239	62
MN, Ca^{++}; then EGTA, pTp after 10 min	1153	1491	23
B. 10 min at 25°C with:			
no addition	2437	3501	≡ 100
Ca^{++}	2367	3498	106
MN	2481	3809	125
Ca^{++}, MN	1118	1450	31
EGTA and pTp added after 10 min			

*Degradation was assayed by formation of cold acid-soluble ^{3}H-oligonucleotides as described under "Materials and Methods." Enhancement by 2',5'-oligo A at 3 X 10^{-8} M was calculated as follows for experiment A: (b-a/1499) 100; and B: (b-a/1064) 100. MN, micrococcal nuclease. Incubation was for 30 min at 30°.

an effect of pTp on the synthesis of the 2',5'-oligo A effector by the synthetase rather than on the nuclease activation function of the effector. To investigate this possibility, the effect on nuclease activation by the addition of dsRNA which activates the synthetase, as compared to the addition of 2',5'-oligo A tetramer which activates the nuclease, was examined. As shown in Fig. 2A, no significant difference was observed in the enhancement of 2',5'-oligo A-dependent nuclease activity by 1 μg/ml dsRNA as compared to 3 X 10^{-8} M 2',5'-oligo A tetramer in nonpreincubated IFN extracts in the presence of pTp.

During the preparation of cell-free protein synthesizing systems dependent upon exogenously added mRNA, endogenous mRNAs are inactivated by a 10 min incubation at 25° with micrococcal nuclease and its required divalent cation, calcium. EGTA and pTp are then added to inactivate the micrococcal nuclease (9-11). As shown in Table 1A, a significant reduction both in the degradation

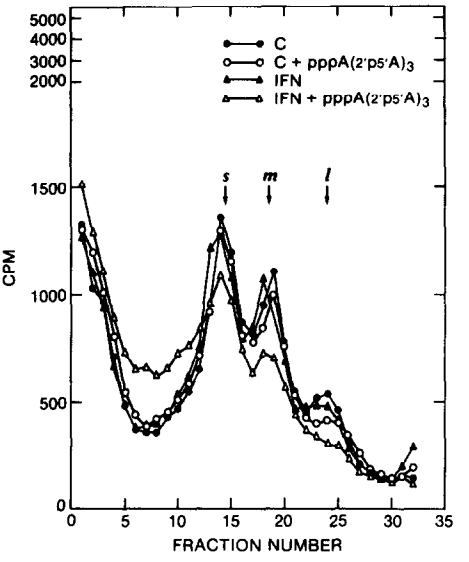

Figure 3. Effect of the interferon treatment on the degradation of reovirus [^{3}H]mRNA in the absence (filled symbols) and presence (open symbols) of 3 X 10^{-8} M pppA(2'p5'A)$_3$. Extracts prepared from untreated (C) and interferon-treated (IFN) cells were treated with micrococcal nuclease and assayed by density gradient centrifugation as described under "Materials and Methods".

of reovirus [^{3}H]mRNA in the absence of 2',5'-oligo A tetramer and in the enhancement in degradation in the presence of tetramer occurred only when the 10 min incubation mixture contained both micrococcal nuclease and calcium. Very little reduction in 2',5'-oligo A nuclease activation was observed after 10 min on ice as compared to 10 min at 25°. These results, obtained by the assay of the formation of acid-soluble ^{3}H-oligonucleotides (Table 1A), were confirmed by sucrose density gradient centrifugation (data not shown). That the presence of active micrococcal nuclease during the 10 min incubation was required for the subsequent reduction in reovirus [^{3}H]mRNA degradation was confirmed by incubation at 25° for 10 min in the absence of both calcium and micrococcal nuclease, or in the presence of calcium alone, micrococcal nuclease alone, or both calcium and micrococcal nuclease prior to the addition of 2',5'-oligo A tetramer. Only when the reaction mixture contained both micrococcal nuclease and calcium was the subsequent degradation of reovirus [^{3}H]mRNA by the activated nuclease reduced (Table 1B).

The effect of 2',5'-oligo A on the degradation of reovirus [^{3}H]mRNA catalyzed by micrococcal nuclease-treated extracts prepared from untreated control as compared to IFN-treated cells was also examined. As shown in Figure 3, sucrose gradient centrifugation profiles of reovirus [^{3}H2mRNA were comparable in extracts from control and IFN-treated cells, both in the presence and absence of 2',5'-oligo A tetramer.

Although the overall extent of mRNA degradation was reduced in nonpreincubated extracts in the presence of either pTp or EGTA (Table 1), the degree of enhancement in degradation by added 2',5'-oligo A tetramer was relatively constant in nonpreincubated extracts over a wide range of concentrations of pTp and EGTA (Figure 2). Active micrococcal nuclease was required to establish the subsequent reduction in 2',5'-oligo A-dependent degradation of reovirus mRNA in extracts supplemented with pTp and EGTA (Table 1). However, the degradation of reovirus mRNA in the absence of 2',5'-oligo A was also reduced in micrococcal nuclease-treated as compared to nonpreincubated extracts supplemented with EGTA and pTp (Fig. 1 and Table 1). Thus, it appears that the 2',5'-oligo A-dependent nuclease as well as other nucleases are inhibited by the oligonucleotide products which accumulate during the digestion of endogenous cellular RNAs by micrococcal nuclease in the preparation of exogenous mRNA-dependent translation systems.

ACKNOWLEDGMENTS

This work was supported in part by research grants from the National Institute of Allergy and Infectious Diseases, N.I.H., and the American Cancer Society. N.G.M. is an Earl C. Anthony Predoctoral Fellow; C.E.S. is a Research Career Development Awardee of the U.S.P.H.S.

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