Double-Stranded Ribonuclease Coinduced with Interferon

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Double-stranded (ds) RNA and many viruses are inducers of interferon (IFN), the latter presumably because they contain, or can form, dsRNA. Concomitant with the induction of IFN in chicken embryo cells was the induction of a novel double-stranded ribonuclease (dsRNase), which was released into the medium and continued to accumulate long after IFN production ceased. Only avian cells (chicken, quail, turkey, or duck) expressed high levels of this dsRNase; mammalian, turtle, or fish cells did not. Production of the nuclease was inducer dose-dependent. Optimum *p*H and cation requirements distinguished it from other dsRNase activities. Degradation of dsRNA was endonucleolytic. Activity resided in a molecule of an M_r of approximately 34,500. Low levels of a single-stranded (ss) RNase activity were inseparable from the dsRNase. The role for a dsRNA-inducible dsRNase released from cells is unknown.

RIBONUCLEASES HAVE BEEN IMPLICATED in IFN action as one facet of its antiviral effects in host defense. The 2′-5′ oligo(A) synthetase–RNase L pathway and its dependence on dsRNA links the action of IFN with dsRNA and reveals the role of a latent endonuclease acting on ssRNA to block viral replication (*1, 2*). Single-stranded RNase activity associated with the membranes of chick embryo cells (CEC) (*3*), and with subviral particles from IFN-treated vesicular stomatitis virus (VSV)–infected cells (*4*) also is implicated in IFN action. We now report that dsRNA and certain viruses (*5–8*), but not IFN, induce in avian cells a secreted soluble nuclease that rapidly degrades dsRNA (dsRNase). Low levels of ssRNase activity associated with this dsRNase appear to explain our earlier report of a membrane-associated alkaline nuclease (*3*). We did not test dsRNAs as substrates at that time nor anticipate the release of nuclease from cells.

Except where noted, all experiments were done with cells from 10-day-old chicken embryos that had been aged in vitro from 7 to 10 days to enhance their capacity to produce IFN (*9, 10*). These CEC then were treated or infected to induce IFN and incubated at 40.5°C in serum-free medium (*9*). Double-stranded RNase released into the medium was measured by determining the rate at which poly(rI) · poly([^{3}H]rC) was rendered acid-soluble under standard reaction conditions initially optimized to detect *Escherichia coli* RNase III (*11*). One unit of dsRNase (or ssRNase) activity was defined as that amount of enzyme that rendered 50% of an RNA substrate acid soluble after 30 min at 37°C in the standard reaction mixture described in the legend to Fig. 1. Units were expressed as the reciprocal of the dilution of enzyme producing this 50% value. Our polyribonucleotide substrate was substantially double-stranded, since high concentrations of pancreatic RNase (30 μg/ml) (Worthington, E.C. 3.1.4.22) added to the standard reaction mixture rendered only 10% of the total ^{3}H-labeled RNA substrate acid soluble after 30 min at 37°C.

The time course of both IFN and dsRNase accumulation in the medium was determined after induction by poly(rI) · poly(rC) (Fig. 1). After a brief lag, IFN accumulated steadily until a peak level was reached by 24 hours; thereafter, IFN activity in the medium decayed slowly. Like IFN, dsRNase was detectable by 4 hours, but accumulated for a longer period than IFN, reaching a peak level at about 72 hours after induction (Fig. 1). In separate experiments, changing the medium every 24 hours after induction demonstrated that IFN production ceased after 24 hours, whereas dsRNase production continued for 48 hours longer, indicating that the dsRNase was not subject to the same temporal regulation that characterizes the coinduced IFN (*12*). Ultraviolet-irradiated Newcastle disease virus (UV-NDV) (*6*) induced a similar time course, but with higher peak yields of IFN and dsRNase, namely, 13,800 and 18,000 units per 10^7 cells, respectively.

IFN-inducers poly(rI) · poly(rC), UV-NDV (6), temperature-sensitive (ts) mutants of VSV (*8, 13*), VSV [±]DI-011 (*5*), and UV-irradiated avian reovirus (*7*) induced accumulation of dsRNase in the medium. The heteroduplexes that do not induce IFN, poly(rI) · poly(dC) (Fig. 1) and poly(dA) · poly(dT), tested over a wide range of concentrations, did not induce IFN or nuclease. Yeast RNA also failed to induce IFN or dsRNase. Active (nonirradiated) NDV induced only small amounts of IFN and dsRNase, even when manifesting a cytopathic effect that released lysosomes and cytoplasmic enzymes into the medium.

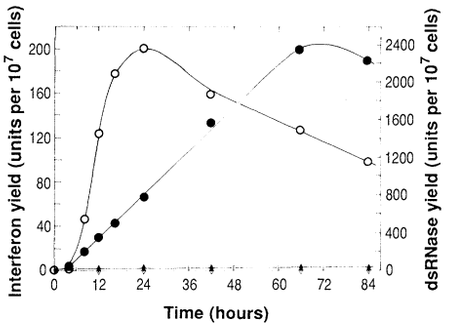

Fig. 1. Time course of interferon (○) and dsRNase (●) accumulation in the culture medium that bathed primary CEC exposed for 1 hour at 37.5°C to a complex of poly(rI) · poly(rC)–DEAE dextran each at 10 μg/ml. Also shown are dsRNase activities from cells treated similarly with poly(rI) · poly(dC)–DEAE dextran (◆) or DEAE dextran only (◇). Results are expressed as units yielded per 10^7 cells. Before treatment the cells were washed three times in serum-free medium to remove serum-derived nucleases from the cell-surface (*30*). Unadsorbed inducer was removed by washing with serum-free medium; 2 ml of the latter was added to each plate, and incubation was at 40.5°C. At various intervals after induction, 10-μl samples were removed from the medium and assayed for IFN (*9*) or dsRNase. The dsRNase activity was measured by adding the 10-μl sample to 40 μl of a reaction mixture containing 50 m*M* tris-HCl, *p*H 8.0; 10 m*M* $MgCl_2$; 100 m*M* NaCl; and, as substrate, poly(rI) · poly([^{3}H]rC) at 2500 cpm. The reaction mixture was incubated at 37°C, and at various intervals samples were withdrawn, added to 0.1% cold yeast RNA, precipitated with 5.0% trichloroacetic acid containing 40 m*M* sodium pyrophosphate, and filtered. The glass-fiber filters were radioassayed for undegraded, acid-precipitable substrate. Units of dsRNase were determined as noted in the text. IFN assay procedures have been described (*9*).

Both IFN and dsRNase were induced in an inducer dose-dependent manner (Fig. 2). Furthermore, the relative amounts of IFN and dsRNase produced varied similarly when inducing conditions were varied: for example, upon changing the concentration of poly(rI) · poly(rC) (Fig. 2), the dose of UV radiation to NDV (*6*), and the duration of aging the cells in vitro (*9, 10*).

Addition of actinomycin D (1 μg/ml) or cycloheximide (50 μg/ml) to the medium during and after virus infection or treatment with poly(rI) · poly(rC) prevented induction of both IFN and dsRNase (*14*), indicating that dsRNase production required new cellular mRNA and protein synthesis. Transcription of the genes required for dsRNase and for IFN production began about 3 hours after virus infection.

Thus far, only avian cells appear to pro-

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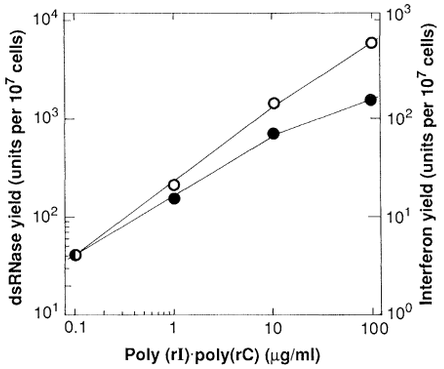

Fig. 2. Poly(rI) · poly(rC) concentration-dependent induction of dsRNase and interferon. Aged monolayers of primary CEC were exposed to poly(rI) · poly(rC) in the presence of 10 μg/ml DEAE dextran. After 1 hour at 37°C the cells were washed three times with serum-free medium and were incubated for 24 hours at 40.5°C. The supernatant medium was then assayed for IFN (●) and dsRNase (○) (see legend to Fig. 1). DEAE dextran controls did not induce IFN or dsRNase (*14*).

duce high levels of a dsRNA-inducible dsRNase. Embryonic cells from turkey, quail, and duck eggs, and *scaleless* mutants of chickens, released similar levels of dsRNase (≃300 units per milliliter) upon induction with poly(rI) · poly(rC). Human FS4, monkey Vero and MA134, mouse L and embryo, brown bullhead fish, and turtle heart cells produced <5% as much dsRNase. Elimination of the liver and pancreas from chick embryos, as possible sources of high nuclease activity (*15*), did not diminish the level of dsRNase-inducible dsRNase obtained from cell cultures prepared from these eviscerated embryos.

All attempts to induce dsRNase with chicken IFN (crude or partially purified) were negative. However, pretreatment of aged chick cells for 24 hours with 10 to 1000 units of IFN, so-called priming (*16*), sometimes enhanced the yield of dsRNase upon induction with poly(rI) · poly(rC).

Poly(rI) · poly(rC) and the genomic dsRNA segments of human reovirus (type 3) were rapidly degraded by the dsRNase activity, whereas heteroduplexes that do not induce IFN, poly(rI) · poly(dC) or poly(dA) · poly(dT), were not sensitive (*14*). All preparations of dsRNase also contained an activity that degraded single-stranded (ss) RNA, albeit at about 5 to 10% of the rate observed for solubilization of dsRNA substrates. Susceptible ssRNA substrates included poly(A), poly(C), the genomic RNAs of encephalomyocarditis and VSV, and the primary transcripts (mRNA) of VSV. Poly(U) was not degraded. Significant ssRNase activity was demonstrated only when dsRNase was induced. The order of susceptibility of ssRNAs to inducible dsRNase, observed as poly(A)>poly(C)>>>poly(U), corresponds to their propensity to assume regions of helicity (*11, 17*).

The nuclease, but not IFN, is inactivated at *p*H 2. The dsRNase, along with the ssRNase, can be separated from IFN by affinity chromatography on agarose–poly(rI) · poly(rC) (Fig. 3), or agarose-poly(rC) (*14*). The ssRNase coeluted with the dsRNase during affinity chromatography, and it too was inactivated at *p*H 2 or when heated for 5 min at 68°C. Both nuclease activities remained in the supernatant fluid after centrifugation for 2 hours at 100,000*g*.

Nuclease activity was dependent on Na^+ and Mg^{2+} or Mn^{2+} (Table 1). Chelation of the divalent cations with EDTA renders the enzyme inactive without affecting its binding to dsRNA, a condition aiding the separation of the enzyme from IFN by affinity chromatography (Fig. 3). The heavy metal ions Hg^{2+} and Zn^{2+} at 1.75 m*M* inhibited more than 90% of the dsRNase activity. Ni^{2+} and Cu^{2+} were partially inhibitory.

The molecular weight of the inducible dsRNase was estimated with a superfine Sephadex G-100 column calibrated with four standard marker proteins: cytochrome c, chymotrypsinogen, ovalbumin, and bovine serum albumin. The dsRNase averaged 34.5 kD in three experiments. Whether this represents a dimeric structure as has been reported for two other dsRNases (*18*) remains to be determined.

The manner in which the inducible dsRNase cleaves dsRNA was determined as follows: ^{3}H-labeled human reovirus genomic dsRNA was reacted with the nuclease, and the resulting products of degradation were analyzed in 7.5% SDS-polyacrylamide gels by electrophoresis. The gels resolved untreated reovirus dsRNA into its characteristic nine bands (ten dsRNA segments), which were revealed after staining with ethidium bromide. These bands were not altered during incubation with material from mock-induced cells. However, by 60 min in the presence of limited amounts of the dsRNase, a large smear of stained material appeared at the lower molecular weight regions of each discrete band. Using highly diluted dsRNase to slow the reaction, we observed substantial diminution in the size of reovirus dsRNA as a function of time, even when only 10% of the ^{3}H-labeled reovirus dsRNA was digested enough to be acid-soluble in our assay. These observations are consistent with an endonucleolytic mode of degradation whereby cleavage across both strands would still yield large pieces of acid-insoluble dsRNA. Exclusive endonucleolytic action in the presence of ssRNase appears to preclude dsRNA unwindase (*19*) in the degradation process, although we have not ruled out exonuclease activity.

Initial studies of dsRNA-induced nuclease were conducted with membrane-associated enzyme, modeled after an earlier report on an ssRNase activity in CEC (*3*). Inducible dsRNase activity, like that of ssRNase, was associated with the fraction of cytoplasmic membranes from the 30 to 40% interface of a discontinuous sucrose gradient (*20*). The membrane-associated and soluble (released) dsRNase activities were similar as judged by the nature of the inducers, induction kinetics, ion requirements, and substrate specificity.

The inducible dsRNase differs from the 2′-5′ oligo(A)–activated endonuclease (RNase L), since the latter is present endogenously in other than avian cells, not inducible, located intracellularly, and only degrades

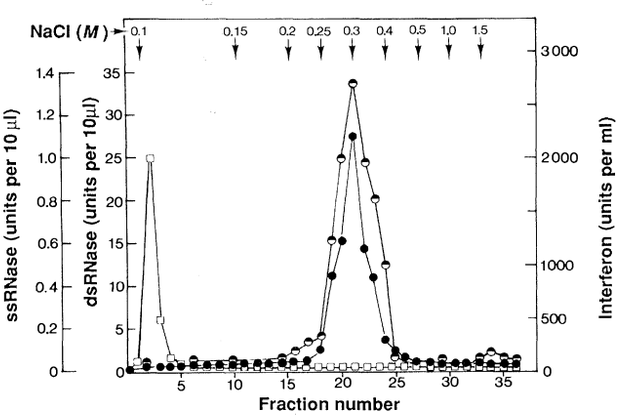

Fig. 3. Separation of interferon and dsRNase by affinity chromatography on agarose–poly(rI) · poly(rC). An agarose–poly(rI) · poly(rC) column (0.75 × 3.5 cm) was equilibrated in buffer containing 50 m*M* tris-HCl, *p*H 8.0; 0.1*M* NaCl; 5.0% glycerol; and 5 m*M* EDTA. Extracellular RNase was prepared from CEC infected with UV-NDV (*6*), and harvested 24 hours after induction. An aliquot was diluted to a final concentration of 0.1*M* NaCl and 5.0 m*M* EDTA and applied to the column. The column was washed with buffer (50 m*M* tris-HCl, *p*H 8.0; 5.0% glycerol; and 5.0 m*M* EDTA) containing increasing concentrations of NaCl. Fractions (1 ml) were collected, and aliquots were tested for IFN (□), dsRNase (◓), and ssRNase (●). Reaction mixtures for the dsRNase and ssRNase substrates, poly(rI) · poly([^{3}H]rC) and poly([^{3}H]rC), respectively, were as described in Fig. 1. Before testing, each fraction was diluted 1:10 into the standard reaction mixture adjusted to equilibrate ionic conditions and compensate for EDTA in the column buffer.

Table 1. Some characteristics of the inducible dsRNase from primary CEC. The dsRNase was induced by infecting cells with UV-NDV (*6*). Before tests for ion requirements, the cell-released nuclease was collected in serum-free medium and centrifuged at 100,000*g* for 2 hours, and the supernatant fluid was desalted through a Sephadex G-25 column with the use of a buffer with 50 m*M* tris-HCl, *p*H 7.4. The dsRNase and ssRNase activities were determined from measurements of the rates of enzymatic conversion of acid-precipitable to acid-soluble amounts of ^{3}H-labeled poly(rI) · poly(rC) or ^{3}H-labeled poly(rC) as substrates, respectively. Ninety percent of the maximum level of activity occurs within the range of values shown in each column. Peak activity occurs at the value shown in parentheses.

RNase	Condition				
	*p*H	NaCl (m*M*)	Mn^{2+} (m*M*)	Mg^{2+} (m*M*)	Heat*
dsRNase	7.5–8.5	0.3–30	0.2–1.6	0.048–1.4	No surviving
	(8.3)	(3)	(0.6)	(0.3)	activity
ssRNase		10–50	1.6–13	1.4–4.6	No surviving
	(8.4)	(30)	(5.1)	(2.6)	activity

*The range of temperatures used—70°, 80°, 90°C for 5 min—tests the possible reacquisition of activity at higher temperatures as was reported for a nuclease from chicken pancreas (*29*).

ssRNA, including poly(U), but not poly(C) (*1, 2*). When *p*H optima, ion requirements, substrate specificities, and molecular weights are compared, the inducible dsRNase appears similar to, but not identical with, only one other dsRNase: DII from CEC (*21*). That enzyme is localized in the nucleus and may play a role in the processing of precursor RNAs (*21*).

Compared to ssRNases (*22*), there are fewer dsRNases (*23*), none of which are reported to be inducible or released from cells. Because this dsRNase is induced by dsRNA and viruses that contain, or produce, dsRNA (*5,24*), we suggest it be designated RNase DS.

The biological significance of a cell-released inducible dsRNase is unknown and warrants further study. Since the antiviral effect mediated by dsRNA is disproportionately large relative to the amount of IFN induced (*5, 24, 25*), it is possible that RNase DS may enhance the action of IFN as an antiviral agent and biological response modifier. Conceivably, it may regulate sense-antisense duplex levels, and thereby gene expression (*26*), or mRNA translation (*27*), especially in the absence of a dsRNA unwindase (*27*).

Note added in proof: Different optima for the induction and assay of RNase DS revealed that mammalian cells also released and accumulated high activities of dsRNA-inducible dsRNA (*28*).

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