Newcastle Disease Virus Stimulates the Cellular Accumulation of Stress (Heat Shock) mRNAs and Proteins

PETER L. COLLINS† AND LAWRENCE E. HIGHTOWER*

Microbiology Section, Biological Sciences Group, The University of Connecticut, Storrs, Connecticut 06268

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A biological agent, Newcastle disease virus, stimulated the synthesis of stress proteins in cultured chicken embryo cells. Previously, only physical and chemical agents were known to induce these proteins. The levels of translatable stress mRNAs were elevated in cells infected with avirulent or virulent strains; however, stress protein synthesis was stimulated strongly only in cells infected by avirulent strains. As did several other paramyxoviruses, avirulent strains of Newcastle disease virus stimulated the synthesis of glucose-regulated proteins as well as stress proteins. Possible stimuli of the synthesis of these two sets of proteins in paramyxovirus-infected cells are considered.

Few known cellular proteins are synthesized at increased rates after infections by negative-strand-RNA viruses. The proteins of the interferon system are the most intensely studied example. In addition, Peluso and co-workers (19) have reported that infection of cultured chicken embryo (CE) cells by the paramyxoviruses Sendai virus and simian virus 5 stimulates the synthesis of several abundant cellular polypeptides. One of these was an 86-kilodalton protein which was not characterized further; the others were identified (20) as glucose-regulated, 99- and 78-kilodalton proteins. Glucose-regulated proteins are cellular proteins synthesized in detectable amounts by uninfected cells under normal culture conditions (14) and at increased rates by mutant cell types (13, 15, 21), during paramyxovirus infection, and in response to glucose starvation (24, 26, 28) and exposure to inhibitors of glycosylation, such as 2-deoxyglucose (21) and tunicamycin (17).

In this report, we show that the accumulation of a second set of proteins and their functional mRNAs in CE cells was stimulated by avian paramyxovirus Newcastle disease virus (NDV) infection. These proteins were identified as the cellular stress (heat shock) proteins. Previously, we (5, 8) and others (9, 11) have demonstrated that stress proteins and their functional mRNAs rapidly accumulate in avian and mammalian cells in response to a variety of stresses, including heat shock, heavy metals, sulfhydryl reagents, amino acid analogs, and tissue explanation. The functions of the stress and glucose-regulated proteins and the mechanisms whereby

they are induced during viral infection are unknown.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to compare [³⁵S]methionine-labeled polypeptides extracted from CE cells infected by the avirulent strains NJ-LaSota (N) (Fig. 1, lane A) and B1-Hitchner (B1) (Fig. 1, lane B) and by the virulent strain Australia-Victoria (AV) (Fig. 1, lane C). The usual complement of previously identified NDV proteins was detected in each case (see references 3 and 25 for complete references). As expected, infection by the avirulent strains did not result in substantial inhibition of host cell protein synthesis characteristic of strain AV (6). Furthermore, cells infected with the avirulent strains contained elevated levels of several size classes of polypeptides (Fig. 1) which appeared to have counterparts in the uninfected control. Peptide mapping by limited proteolysis (data not shown) confirmed that these polypeptides were present at lower levels in the uninfected control. Coelectrophoresis of polypeptides extracted from strain N-infected CE cells (Fig. 2, lane A) and authentic glucose-regulated proteins from 2-deoxyglucose-treated CE cells (lane C) showed that two of the virus-stimulated polypeptides (M_r s = 99,000 and 78,000) had the expected electrophoretic mobilities of the glucose-regulated proteins. The large glucose-regulated protein accumulates in an underglycosylated, 97-kilodalton form in the presence of 2-deoxyglucose (20, 22) and therefore migrated slightly ahead of the 99-kilodalton, virus-stimulated polypeptide. Peptide mapping by limited proteolysis (data not shown) confirmed the identification of the 99- and 78-kilodalton, virus-stimulated polypeptides as the glucose-regulated proteins.

† Present address: Department of Bacteriology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, NC 27514.

FIG. 1. Comparison by SDS-PAGE of polypeptides extracted from NDV-infected and uninfected CE cells. Secondary cultures of CE cells were infected (multiplicity of infection, 5) with NDV strains N (lane A), B1 (lane B), and AV (lane C) or were mock infected (lane D). The cultures were then incubated for 6 h, exposed to 50 μ Ci of [35 S]methionine per ml of medium for an additional 0.5 h, solubilized, and analyzed by using an 11.5% polyacrylamide gel and fluorography. The positions of the viral polypeptides (L, HN, F₀, NP, F₁, P, and M) were marked according to current nomenclature (see reference 25 for references). The positions of virus-stimulated cellular polypeptides were marked by their approximate molecular sizes in kilodaltons (5, 20). Preparation of secondary cultures of CE cells, virus purification, conditions of infection, radioisotopic labeling, and SDS-PAGE have been described previously (3).

The remaining NDV-stimulated polypeptides (labeled in Fig. 2, lane A, bands marked 88, 72, and 71) comigrated with stress proteins extracted from CE cells exposed to the arginine analog canavanine (Fig. 2, lane E). In some infections, 34- and 23-kilodalton, virus-stimulated polypeptides which also comigrated with known stress proteins were detected. Stimulated synthesis of the 110-kilodalton stress protein was not detected during viral infections. Limited-digest peptide mapping established that NDV-stimulated proteins and stress proteins with similar electrophoretic mobilities had identical peptide maps (Fig. 3).

In stressed uninfected cells, the rapid accumulation of stress proteins is accomplished by increases in the rate of protein synthesis rather

than by changes in degradation rates (5), and increased cytoplasmic levels of functional stress mRNAs have been demonstrated (8, 9, 11). Similarly, radioisotopic pulse-chase experiments indicated that the response in NDV-infected cells was not due to alterations in polypeptide stability (data not shown). Therefore, it was of interest to compare the levels of functional cytoplasmic mRNAs in mock-infected cells with those in NDV-infected cells by mRNA extraction, purification, and translation *in vitro*. Analysis of the translation products by SDS-PAGE (Fig. 4) demonstrated that NDV-infected cells contained elevated levels of mRNAs encoding the glucose-regulated and stress proteins and that the levels of translatable stress mRNAs

FIG. 2. SDS-PAGE of stress proteins, glucose-regulated proteins, and proteins extracted from NDV-infected cells. The polypeptides were from CE cells that had been infected (multiplicity of infection, 5) with NDV strain N and incubated for 9.5 h at 40.5°C (lane A), infected with strain N as in lane A except that actinomycin D (2 μ g/ml) was added at 3.25 h postinfection (lane B), treated with the glycosylation inhibitor 2-deoxyglucose (10 mM) for 24 h (lane C), mock treated (lane D), or treated with L-canavanine (0.6 mM) in arginine-free medium for 3 h (lane E). All of the cultures were incubated for an additional 0.5 h in the presence of [35 S]methionine in arginine-containing medium. The cultures were then solubilized and analyzed by SDS-PAGE on 9% polyacrylamide gels. The positions of viral, glucose-regulated, and stress proteins (designated by size in kilodaltons) are marked in the resulting autoradiogram. The exact conditions for treatment of CE cells with canavanine and 2-deoxyglucose have been described previously (5).

approached those of viral mRNAs. In control experiments, we determined that the translation of stress mRNAs *in vitro* was inhibited by the addition of AV mRNAs to the translation system

FIG. 3. Comparison by limited-digest peptide mapping of virus-stimulated cellular polypeptides with authentic stress proteins. [35 S]methionine-labeled proteins were separated by SDS-PAGE on gels such as those shown in Fig. 1, 2, and 4, excised from the dried gels, and analyzed by limited protease digestion in conjunction with SDS-PAGE (2). *Staphylococcus aureus* protease V-8 was used at concentrations of 1 μ g/ml (lanes A through D) and 25 μ g/ml (lanes E through H). The left lane of each pair is the canavanine-stimulated stress protein, and the right lane is the NDV-stimulated counterpart from infected cells. The maps are of the 88-, 72-, 71-, and 23-kilodalton stress proteins (lanes A and E, B and F, C and G, and D and H, respectively). In this particular experiment, the [35 S]methionine-labeled polypeptides were synthesized in reticulocyte lysates programmed with polyadenylate-containing cytoplasmic mRNA from canavanine-treated and NDV strain B1-infected cells (2, 3, and 5; legend to Fig. 4). The same results were obtained with polypeptides synthesized in infected cells (data not shown).

FIG. 4. SDS-PAGE of polypeptides synthesized in reticulocyte lysates programmed with mRNAs from infected and uninfected CE cells. [35 S]methionine-containing polypeptides were synthesized *in vitro* in response to mRNAs from cells infected with strain AV (lanes A and B) or strain B1 (lanes C and D) and mock infected CE cells (lanes E and F). The cultures represented in lanes B, D, and F received 2 μ g of actinomycin D per ml of culture medium at 3.25 h postinfection; the other cultures received no drugs. Cytoplasmic extracts were prepared at 10 h postinfection, and the polyadenylate-containing mRNA was selected with columns of oligodeoxythymidylic acid-cellulose. The products of cell-free translation of the mRNA preparations were analyzed on a 9% polyacrylamide gel and processed by fluorography. The band marked HN₆₇ was the nonglycosylated form of HN from strain B1; the strain AV nonglycosylated form synthesized in these cell-free extracts had a slightly lower mobility and partially overlapped the 71-kilodalton stress protein. The M protein of strain AV also had a lower mobility than that of strain B1. The exact conditions for mRNA purification and cell-free translation have been described previously (2, 3).

(data not shown). Thus, the increased synthesis of stress proteins in NDV-infected cells (Fig. 1 and 2) and in reticulocyte lysates programmed with mRNA from NDV-infected cells (Fig. 4) was not due to the preferential translation of a small population of cellular mRNAs but instead reflected a genuine and substantial increase in the accumulation of functional stress mRNAs.

The addition of actinomycin D at 3.25 h postinfection blocked the increased accumulation of stress proteins (Fig. 2, lane B) and their functional mRNAs (Fig. 4, lanes B and D). The simplest interpretation of these data is that the stress response was due to increased cellular gene transcription initiated after 3.25 h postinfection. However, posttranscriptional modulation of mRNA levels was not rigorously excluded as a possibility.

The relative amounts of 88-, 72-, and 71-kilodalton stress proteins synthesized in reticulocyte lysates in response to mRNAs extracted from AV-infected cells (Fig. 4, lane A) and B1-infected cells (lane C) were approximately the same. We concluded that the amounts of functional stress protein mRNAs increased after infections by avirulent and virulent strains, but in the latter case, stress protein mRNA translation was inhibited, along with the translation of most other cellular mRNAs. The previously observed (7) apparent resistance of 88-kilodalton stress protein synthesis by strain AV-infected cells to inhibition (Fig. 1, lane C) was possibly a consequence of the relatively large amount of the mRNA corresponding to the protein (Fig. 4, lane C).

We conclude that the biological agent NDV, as well as thermal stress and chemicals, can induce the major stress proteins in avian cells. It is likely that Sendai virus and simian virus 5 each induce at least one stress protein in CE cells, since the 86-kilodalton protein observed by Peluso et al. (19) in Sendai virus-infected cells (obtained from R. A. Lamb, The Rockefeller University, New York) has the same electrophoretic mobility as that of our 88-kilodalton protein (L. E. Hightower, unpublished data). The 72- and 71-kilodalton stress proteins may have been masked by the Sendai virus HN glycoprotein ($M_r = 70,000$) in other studies (19, 20). The *src* protein of Rous sarcoma virus binds to the 88-kilodalton stress protein, but retroviral infection does not stimulate the protein's synthesis (1, 18).

Paramyxoviruses are probably not unique among viruses in causing stress to the host cell. However, the full expression of stress proteins is likely to be blocked during infections by viruses which rapidly inhibit host macromolecular synthesis, such as virulent strains of NDV. Thus, the response at the protein level is more likely to be observed during infections by avirulent viruses or less cytopathic mutants of virulent viruses and during persistent infections. Interestingly, when *Escherichia coli* is infected by bacteriophage λ , the synthesis of *groE* protein is stimulated, whereas the syntheses of most cellular proteins are inhibited (4, 12). *groE* protein is required for the assembly of bacteriophage λ , and it has recently been identified as an

E. coli heat shock protein (see references 16 and 27 for complete references).

The mechanism by which the syntheses of stress and glucose-regulated proteins are stimulated by paramyxoviruses is not known. Previously, we have suggested that the cellular accumulation of abnormal proteins or their degradation products may be a trigger for the induction of stress proteins (5). It is possible that NDV infection also causes cells to accumulate aberrant proteins or that certain NDV proteins are recognized by the cell as abnormal. Peluso et al. (20) showed that the stimulated accumulation of glucose-regulated proteins in paramyxovirus-infected cells is not due to increased glucose utilization and depletion. Peluso et al. postulated that cell membrane changes, possibly caused by viral glycoproteins, are involved. In this context, we note that the NDV HN glycoprotein accumulates to high levels in the Golgi or pre-Golgi segment of its maturation pathway, as well as on the cell surface (23). If the synthesis or intracellular accumulation of HN interferes with cellular glycoprotein synthesis and migration, then viral infection could mimic the effects of glycosylation inhibitors in triggering glucose-regulated protein synthesis.

The following additional evidence of a link between the inductions of glucose-regulated and stress proteins has been obtained recently. (i) Treatment of cultured rat embryo cells with the arginine analog canavanine results in stimulated syntheses of both groups of proteins (8; L. E. Hightower and F. P. White, manuscript in preparation). (ii) Both sets of proteins rapidly accumulate in mammalian tissue explants (Hightower and White, manuscript in preparation). (iii) The synthesis of a protein that is apparently analogous to the 88-kilodalton stress protein is inhibited in glucose-deprived mouse L cells (10). Further studies are needed to evaluate possible relationships between these intriguing sets of proteins.

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ADDENDUM IN PROOF

J. R. Nevins has reported the induction of a heat shock protein by adenovirus-5 (Cell 29:913-919, 1982), and E. L. Notarianni and C. M. Preston have reported the activation of genes encoding cellular stress proteins during infections by mutants of herpes simplex virus type 1 (Virology, in press).

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