Brief Definitive Report

Protein Kinase Regulates Tumor Necrosis Factor mRNA Stability in Virus-stimulated Astrocytes

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Summary

Infection of astrocytes with Newcastle disease virus stimulated the production of 1,2-diacylglycerol, and resulted in the kinase-dependent expression of mRNAs encoding tumor necrosis factor (TNF), interferon α and β, and interleukin 6. The half-life of TNF mRNA was significantly decreased in the presence of protein kinase inhibitors H-7 and staurosporine, but not in the presence of HA1004. In contrast to the decay of TNF mRNA, the half-lives of other cytokine mRNAs were only minimally affected by the kinase inhibitors. These data indicated that the stability of TNF mRNA was regulated through a novel, kinase-dependent pathway.

Immunologically competent cells have the capacity to regulate expression of cytokine genes. Much attention has been focused on *cis*-acting promoter sequences that serve as binding targets for the diverse factors that influence transcription. Of potentially equal importance is the regulated control of mRNA stability. Although relatively little is known of the factors that influence mRNA half-life in eukaryotic cells, signals generated at the plasma membrane may alter the stability of certain mRNAs, including those encoding cytokines (1–3). Stabilization of these transiently expressed mRNAs can result in higher levels of secreted protein (1, 3). Cytokine and proto-oncogene mRNAs, which are among the least stable eukaryotic RNAs, contain an AU-rich element in their 3′ untranslated regions that is similar to a sequence from granulocyte-macrophage (GM)-CSF mRNA that confers instability upon β-globin mRNA (4). In addition, multiple decay mechanisms appear to concurrently regulate the stability of these short-lived mRNAs, although the signal messengers and recognition sequences comprising these pathways are poorly defined.

Along with their well-defined roles as immunomodulators, the cytokines encoded by these mRNAs may also influence glial cells and neurons within the central nervous system (CNS). Interestingly, we have observed that recombinant TNF/cachectin kills primary oligodendrocytes, cells that form the myelin sheath (5). Since an infectious agent such as a virus may play a role in the development of primary demyelination, we previously examined whether TNF was produced after virus infection of astrocytes. Astrocytes infected with Newcastle disease virus (NDV) produced TNF as well as IFN-α, IFN-β, and IL-6 (6). In this report, we examined the mechanism by which virus stimulated cytokine gene expression in astrocytes, and observed that the accumulation of TNF mRNA was mediated through a novel, kinase-dependent mRNA stabilization pathway.

Materials and Methods

Cell Culture and Stimulation. Primary cultures of rat astrocytes were established as described (5). Approximately 95% of the cells expressed the astrocytic marker glial fibrillary acidic protein and <2% expressed MAC-1. For induction, cells were stimulated with NDV, New Jersey LaSota strain, at a multiplicity of 30.

Measurement of 1,2-diacylglycerol. Astrocytes (8×10^5/60-mm dish) were incubated in serum-free DMEM/Ham's F12 for 30 min at 37°C and then stimulated with NDV. Reactions were terminated by the addition of ice-cold methanol. Measurement of cellular mass levels of 1,2-diacylglycerol and lipid phosphorus were performed as described (7). Data are reported as nanomoles of 1,2-diacylglycerol per 100 nmol of lipid phosphorus. There were ~55 nmol of lipid phosphorus per 8×10^5 cells.

Northern Blot Analysis. Total RNA was isolated and analyzed as described (6). Labeled RNA probes for mouse cytokines and IFN regulatory factor 1 (IRF-1) were generated using SP-6 and T7 promoter vectors. The TNF probe was constructed from a 1.1-kb fragment of TNF cDNA, the IFN-β probe from a 500-bp PvuII fragment of IFN-β cDNA, the IFN-α_4 probe from a 776-bp EcoRI-BglII fragment of the IFN-α_4 genomic clone, and the IRF-1 probe from a 1,024-bp XbaI-PvuII fragment of the IRF-1 cDNA. DNA probe for IL-6 was constructed using an oligolabeling reaction kit (Pharmacia Fine Chemicals, Piscataway, NJ) and a 650-bp EcoRI-BglII fragment of IL-6 cDNA.

Nuclear Run-On Assay. Nuclei (3×10^7) were isolated and nascent transcripts elongated in vitro as described (8). Labeled RNA was hybridized to denatured plasmids containing inserts for TNF, IRF-1, and β-actin that had been immobilized on nitrocellulose filters. pSV2-neo served as a control for nonspecific hybridization.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/90/09/0989/04 $2.00
Volume 172 September 1990 989–992

Table 1. *1.2-Diacylglycerol Production by Astrocytes Stimulated with NDV*

Stimulant	Time	DAG
	min	*mole %*
Unstimulated	0	0.460 ± 0.215
NDV	1	1.169* ± 0.450
NDV	5	0.757 ± 0.458
NDV	10	0.579 ± 0.160
NDV	20	0.613 ± 0.259

* Value is statistically different from unstimulated sample; $p < 0.005$ by unpaired student's t test.

Results and Discussion

We began to explore the mechanism by which NDV induces cytokine mRNA accumulation by examining the role of signal messengers generated after cells were exposed to virus. NDV stimulated a transient production of 1,2-diacylglycerol in astrocytes that peaked within 1 min (Table 1). To determine whether activated protein kinases, particularly protein kinase C (PKC), participated in cytokine mRNA accumulation, cells were infected with NDV in the presence or absence of kinase inhibitors (Fig. 1). Infection of astrocytes with NDV in the presence of H-7 or staurosporine, two compounds that inhibit PKC (9, 10), prevented the accumulation of TNF, IFN-α, IFN-β, and IL-6 mRNAs in a dose-dependent fashion. In contrast, HA1004, a compound structurally related to H-7 but a relatively poor PKC inhibitor (9), failed to block cytokine mRNA accumulation. These results indicated that NDV-induced accumulation of cytokine mRNAs required PKC activity. Interestingly, accumulation of mRNA encoding IRF-1 was not affected by the kinase inhibitors. IRF-1 is a virus-inducible DNA binding protein that plays a positive role in the expression of IFN-β and possibly other cytokines (11). This finding confirmed that treatment of astrocytes with H-7 and staurosporine did not cause a nonspecific block of mRNA induction, and demonstrated that overexpression of IRF-1 mRNA can be dissociated from virus-induced expression of cytokine mRNAs.

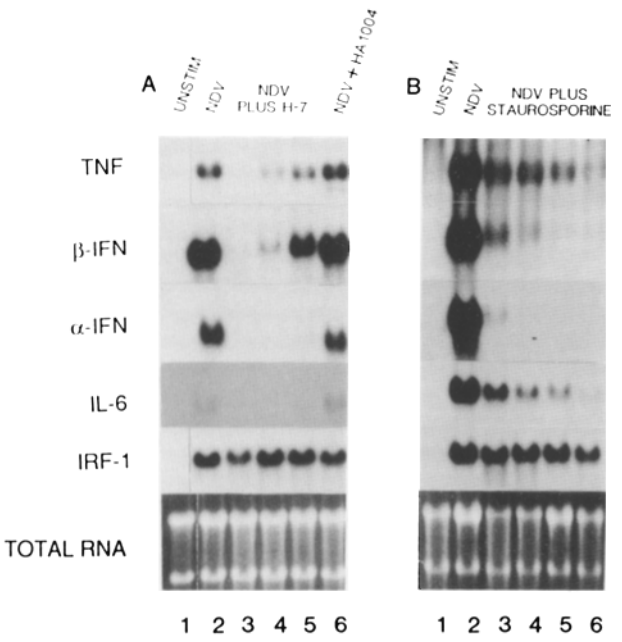

Figure 1. Effect of kinase inhibitors on cytokine mRNA accumulation after NDV infection. Astrocytes were preincubated for 30 min at 37°C in the presence or absence of kinase inhibitors, and then infected with NDV in the continuous presence of the kinase inhibitors. Total RNA, collected (*A*) 8 h or (*B*) 6 h after infection, was analyzed by Northern blot (10 μg/sample). (*A*) Unstimulated, lane *1*; NDV stimulated, lane *2*; NDV plus H-7 (60, 30, and 15 μM), lanes *3–5*; NDV plus HA1004 (60 μM); lane *6*. (*B*) Unstimulated, lane *1*; NDV stimulated, lane *2*; NDV plus staurosporine (0.25, 0.50, 0.75, and 1.0 μM), lanes *3–6*.

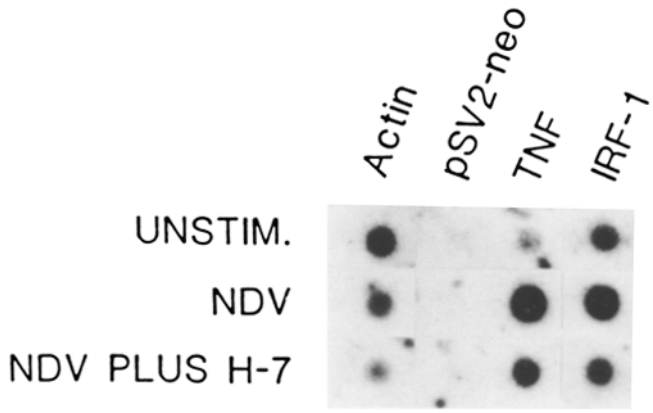

Figure 2. Effect of H-7 on NDV-stimulated transcription. Run-on assays were performed with nuclei isolated from unstimulated astrocytes, and from cells stimulated for 4 h with NDV in the presence or absence of H-7 (60 μM). Cells infected in the presence of H-7 were preincubated with the kinase inhibitor for 30 min at 37°C.

To determine whether the kinase inhibitors affected NDV-stimulated transcription, run-on assays were performed with nuclei isolated from unstimulated astrocytes, and from cells stimulated with NDV or NDV in the presence of H-7 (Fig. 2). Virus infection increased TNF and IRF-1 transcription and concomitantly decreased actin transcription. A noncytotoxic dose of H-7 caused a partial inhibition of NDV-induced transcription of TNF and IRF-1. Although the transcription of these two genes was inhibited to a similar degree, H-7 had strikingly different effects on TNF and IRF-1 mRNA accumulation (Fig. 1). These findings suggested to us that a pathway involving protein kinases may regulate the stability of TNF mRNA in NDV-stimulated cells.

The role of kinases in regulating cytokine mRNA half-life was examined by infecting astrocytes with NDV for 6–8 h, and then adding α-amanitin at a concentration that blocked transcription in the presence or absence of kinase inhibitors. Assessment of mRNA decay by Northern analysis (Fig. 3) revealed that all of the cytokine mRNAs induced by virus decayed with a relatively long half-life of several hours. Both H-7 and staurosporine inhibited the virus-induced stabiliza-

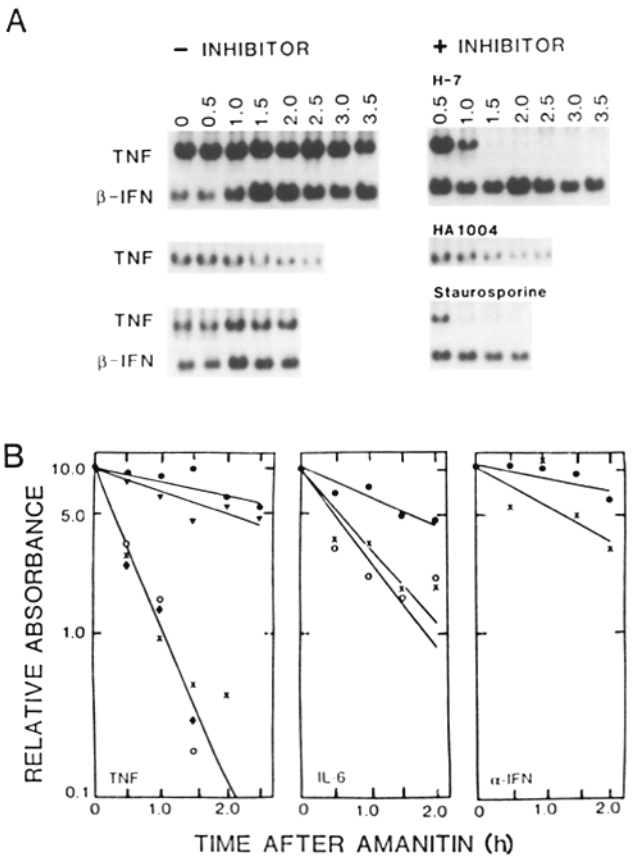

Figure 3. Influence of kinase inhibitors on the decay of cytokine mRNAs. (*A*) Astrocytes were infected with NDV for 6 or 8 h, and then treated with α-amanitin (5 μg/ml) in the presence or absence of the kinase inhibitors H-7 (60 μM), HA1004 (60 μM), or staurosporine (1 μM). Total RNA, collected at the time of α-amanitin addition ($t = 0$) or after further incubation, was analyzed by Northern blot (10 μg/sample). (*B*) Summary of cytokine mRNA decay in the presence or absence of kinase inhibitors. mRNA accumulation was quantitated by laser densitometry and standardized to the intensity of β-actin mRNA. α-amanitin alone (●); α-amanitin in the presence of: H-7 (○); staurosporine (x); HA1004 (▼); and H-7 and CHX (15 μg/ml) (◆).

tion of TNF mRNA, whereas HA1004 did not. Densitometric analysis of blots reprobed and standardized for β-actin mRNA demonstrated that TNF mRNA stability decreased ~10-fold (from 180 ± 52 min to 17 ± 0.9 min) in the presence of H-7 or staurosporine. Simultaneous treatment of astrocytes with cycloheximide (CHX) plus H-7 also resulted in rapid TNF mRNA decay, suggesting that neither transcription nor ongoing translation was required for degradation. In contrast to the decay of TNF mRNA, the half-lives of IFN-β, IFN-α, and IL-6 mRNAs were minimally affected by H-7 or staurosporine, consistently decreasing two- to threefold. The stability of IRF-1 mRNA was also minimally affected by H-7 (data not shown).

We have demonstrated that TNF mRNA was stabilized through a novel, kinase-dependent pathway after virus infection. This pathway profoundly influenced TNF mRNA accumulation, and may therefore play an important role in the regulation of TNF protein synthesis. All of the cytokines examined, as well as IRF-1, contain AU-rich element in the mRNA 3′ untranslated region (4, 11), suggesting that this destabilizing sequence may not be a sufficient signal to preferentially target TNF mRNA to the kinase-modulated regulatory pathway. Stabilization of other cytokine mRNAs induced in astrocytes by NDV was predominantly mediated through distinct, PKC-independent mechanisms.

Signal messengers have been previously implicated in the stabilization of cytokine mRNAs. Stimulation of transcriptionally activated T cells with anti-CD28 stabilizes cytokine but not proto-oncogene mRNAs (3). Both LPS and phagocytosis increase GM-CSF mRNA accumulation in macrophages by a post-transcriptional mechanism (1). LPS also acts as a post-transcriptional stimulator for TNF production by macrophages, increasing both mRNA accumulation and translation (2, 12). Although specific sequences involved in the regulation of stability have been identified in transferrin receptor, histone, and β-tubulin mRNAs (13–15), the elements involved in these cytokine regulatory pathways are currently unknown. Further work will help to clarify the sequences in TNF mRNA that may act as targets for the kinase-modulated stabilization pathway.

We thank the following for their gifts of plasmids used to construct cytokine probes: B. Raj, type I IFN; B. Beutler, TNF; J. vanSnick, IL-6; and T. Taniguchi, IRF-1. We also thank Beth Drysdale for assistance with the measurement of DAG, and D. Cleveland, D. Fearon, and H. Shin for critical review of this manuscript.

This work was supported by grants RO1-NS-15662, NS-20022, (M. L. Shin), and RO1-AI-19737 (P. M. Pitha) from the National Institutes of Health; and PP0084 from the National Multiple Sclerosis Society.

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Received for publication 12 April 1990 and in revised form 11 June 1990.

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