to 96-well plates (Nunc 1) that had been coated with goat polyclonal antibodies to NGF (specific) or normal goat serum (nonspecific). The immobilized NGF was then incubated with monoclonal antibody 23C4 to β-NGF, and the complex was visualized by sequential reactions with biotin-conjugated goat antibody to rat IgG, horseradish peroxidase–conjugated strept-avidin, and the peroxidase substrate ortho-phenylenediamine. The absorbance of the wells was read at 492 nm. The amount of NGF in individual samples was calculated by comparison with β-NGF purified from mouse submaxillary gland. The sensitivity of the assay, defined as two times the background signal, was routinely 0.1 to 1 pg of NGF per assay. Recovery of NGF from the neocortex and hippocampus, determined by addition of purified mouse β-NGF to the homogenates followed by ELISA, was greater than 75%. The data were not corrected for recovery.

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An Early Event in the Interferon-Induced Transmembrane Signaling Process

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Human interferon stimulates a transient two- to threefold increase in the concentration of diacylglycerol and inositol tris-phosphate within 15 to 30 seconds of cell exposure to interferon. Antibodies to interferon inhibit this effect. The stimulation was measurable in isolated cell membranes exposed to interferon. Human α and β, but not γ, interferon stimulate this increase in cells containing the appropriate interferon receptor. The effect was proportional to the number of interferon receptors. Both the diacylglycerol increase and antiviral effects induced by interferon could be correlated in terms of dose dependence. Thus, a transient diacylglycerol increase is an early event in the interferon-induced transmembrane signaling process.

INTERFERON (IFN) IS ANTIVIRAL, HAS immunoregulatory effects in living cells, and inhibits cell growth (*1*). Clinically, it is an effective prophylactic agent against the common cold (*2*) and has an antiviral effect in other viral diseases of man such as warts, hepatitis B, and reactivated herpes simplex (*3*). It is reported to have an antitumor effect in hairy-cell leukemia, juvenile laryngeal papilloma, and intraepithelial neoplasia of the uterine cervix (*4*). However, little is known of the physiological role of IFN and of the mechanism by which IFN transmits its effects from the cell surface into the cell where its actions are manifested. We undertook to study the early events that occur in cells seconds after they are exposed to IFN to identify the changes associated with the transmembrane processing of the IFN signal. For this reason, we chose to study primary human diploid fibroblasts and Daudi cells because they are most commonly used in IFN assays and in studying the binding of human IFN's α and β to its putative receptor (*5*).

Primary human fibroblasts, Daudi cells, and isolated cell membranes were incubated at 37°C with human IFN's α, β, or γ for various times. The cells were immediately extracted and assayed for diacylglycerol and inositol phosphates. The treated cells were also assayed for changes in cytosolic-free Ca^{2+} by means of quin-2 and fura-2 as fluorescent indicators (*6*). There were immediate increases in diacylglycerol and inositol bis- and tris-phosphates in human fibroblasts within 30 seconds after exposure to human IFN's α, β, and γ (Fig. 1A). The concentration of diacylglycerol returned to basal level within 30 to 60 seconds. The concentrations of inositol bis- and tris-phosphates returned to basal levels by 10 minutes. Similar results were observed in Daudi cells except that the rise in diacylglycerol concentration was faster in cells treated with human IFN α than with human IFN β (Fig. 1B). The kinetics of this response to human IFN α was about 15 seconds faster than those of IFN β. A statistically significant increase (49%) in diacylglycerol was observed in isolated Daudi cell membranes exposed to human IFN β (Table 1), suggesting that the increase in diacylglycerol concentration occurs in the cell membrane

Table 1. Diacylglycerol levels in different cell types and in cell membranes exposed to human IFN's α, β, and γ; N.D., not determined.

Cells* or membranes†	Diacylglycerol (% of control) after treatment with IFN		
	α	β	γ
Human diploid fibroblasts	280 ± 21‡	281 ± 25‡	343 ± 14‡
Daudi cells	202 ± 3‡	352 ± 11‡	98 ± 2
Mouse A_9 cells	104 ± 7	111 ± 21	96 ± 7
Mouse A_9 × human WaV hybrid	146 ± 2‡	156 ± 13‡	102 ± 2
Mouse A_9 × human WaIII hybrid	N.D.	101 ± 31	N.D.
WaV subclone (WaVR4dF94a)	187 ± 13‡	201 ± 6‡	97 ± 1
Daudi cell membranes	N.D.	149 ± 12‡	N.D.

*Fibroblast cells were grown to confluency (2×10^4 to 4×10^4 per square centimeter) in 75-cm^2 flasks. Daudi cells in 5-ml suspensions were at a density of 1×10^6 cells per milliliter. The cells were exposed to human IFN's α, β, and γ (4000 IU/ml) for 30 seconds and then extracted and assayed for diacylglycerol by the method of Habenicht *et al.* (*14*). †Membranes from Daudi cells (5×10^6) prelabeled with [2-^{3}H]glycerol and prepared by the method of Lucas *et al.* (*19*) were incubated for 10 minutes at 37°C with human IFN β (10,000 IU/ml). ‡Indicates that the value shown is statistically different from the untreated control by Student *t* test at $P < 0.05$. Each value is the mean ± SD of duplicates.

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and is possibly produced by IFN-receptor interactions. It is unlikely that these increases require internalization of the receptor-IFN complex because internalization (*7*) occurs after the onset of these early events.

To show that the IFN receptor is involved, diacylglycerol concentration changes were measured in cell types known to respond differently to human IFN's by virtue of the presence of the appropriate receptors. Human diploid fibroblasts contain the receptors for both human IFN's α and β and IFN γ. These cells responded with rapid and transient increases in diacylglycerol and inositol bis- and tris-phosphates when exposed to any of these IFN's (Fig. 1A and Table 1). Daudi cells bind human IFN's α and β but not IFN γ specifically. They responded only to human IFN's α and β but not IFN γ (Fig. 1B and Table 1). Mouse A_9 cells, which do not have receptors for human IFN, did not show an increase in diacylglycerol concentration when exposed to either human IFN's α and β or IFN γ (Table 1). Mouse-human hybrid cells (WaV) that have chromosome 21 as one of four human chromosomes (*8*), and a WaV subclone (WaVR4dF94a) that has chromosome 21 (*9*) as its only human chromosome responded to human IFN's α and β but not IFN γ (Table 1). A hybrid clone (WaIII) that has lost chromosome 21 (*8*) did not respond to human IFN β (Table 1). Polyclonal antibodies to IFN β, which are known to neutralize the biological activity of IFN β by inhibiting its binding to its receptor (*10*), also inhibited the IFN-induced increases in diacylglycerol and inositol phosphates (Figs. 1 and 2). These results suggest that a rise in diacylglycerol concentration in cells exposed to IFN's α and β requires IFN to react with its receptor.

Genes encoding the putative human IFN α and β receptors have been localized on chromosome 21 (*8, 11*). A dosage relationship was established between the antiviral effect of IFN's α and β and the number of copies of chromosome 21 in human fibroblasts (*9*). The concentration of IFN required to fully stimulate an increase in diacylglycerol in primary human fibroblasts containing one, two, and three copies of chromosome 21, respectively, and hence having more receptor for IFN's α and β (*11*), was measured. The concentration of IFN's α and β required to stimulate a maximal increase in diacylglycerol concentration in cells containing one, two, and three copies of human chromosome 21 was 24,000, 6,000, and 1,500 IU/ml, respectively (Table 2). This inverse relationship suggests that the increase in diacylglycerol in IFN-treated cells is dependent on the interaction of IFN with its putative receptor.

To show that the stimulation of diacylglycerol increase in IFN-treated cells is relevant to the antiviral effect of IFN, human diploid fibroblasts were exposed for 30 seconds at 37°C to different concentrations of human IFN β (Fig. 2). The IFN was removed by washing with a fivefold excess of antibodies to IFN and the cells were tested for their ability to support viral replication under the conditions described (*12*) or immediately assayed for diacylglycerol. The antiviral effect of IFN correlated closely to the diacylglycerol increase in cells exposed to different concentrations of IFN (Fig. 2). IFN concentrations that did not produce an increase in diacylglycerol also did not induce the antiviral state. This, together with the genetic data, suggests that the rapid and transient rise in diacylglycerol concentration could be associated with the antiviral activity of IFN.

IFN also stimulates a transient rise (40 to 240%) in inositol tris-phosphate and a smaller increase (15%) in inositol bis-phos-

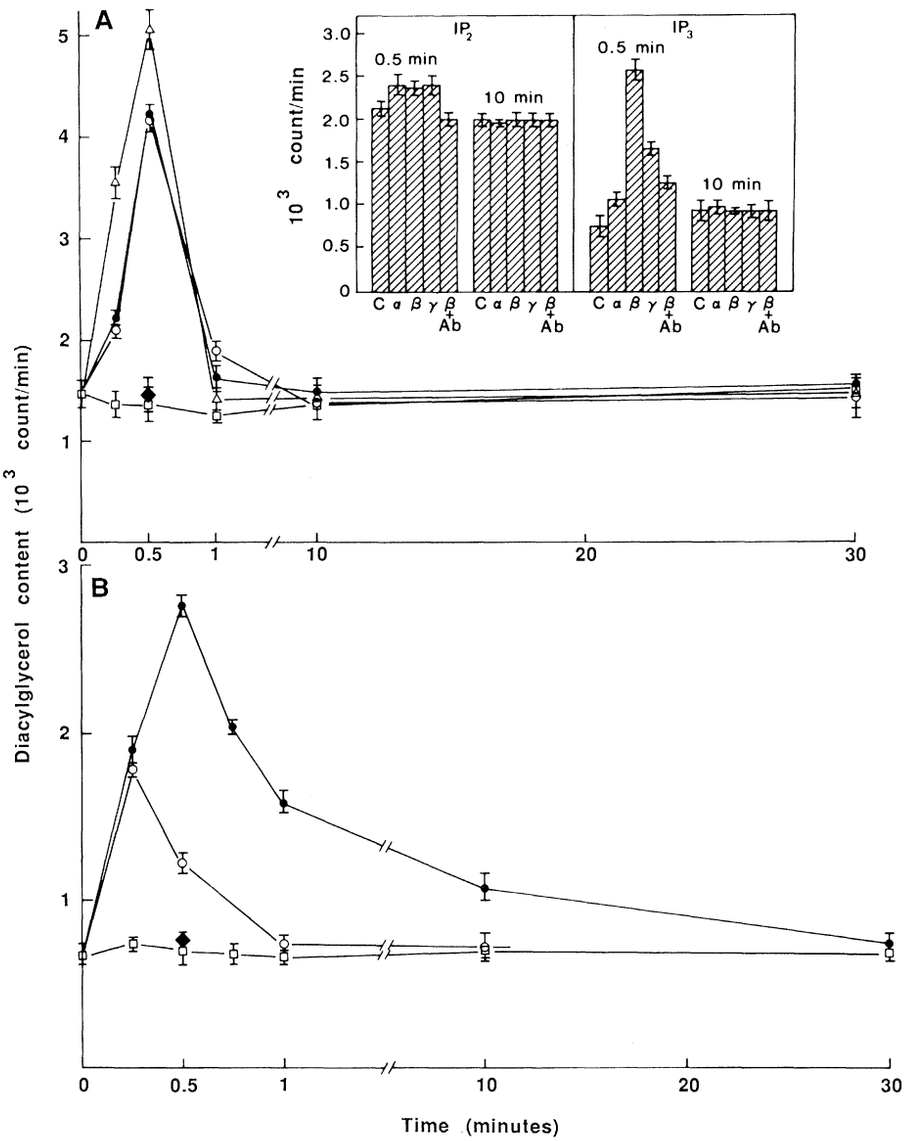

Fig. 1. (A) The kinetics of IFN-induced changes in diacylglycerol levels in human diploid fibroblasts. IFN concentration was kept at 20,000 IU/ml. The cells were exposed to IFN's α (○), β (●), and γ (△) for the times indicated. The diacylglycerol level in control fibroblasts is indicated by (□) and in fibroblasts exposed to a mixture of human IFN β and a twofold excess of rabbit polyclonal antibodies to human IFN β is indicated by (♦). The levels of inositol bis- (IP_2) and tris- (IP_3) phosphates are indicated by the histograms in the inset, where C, α, β, γ, and β + Ab denote control cells and cells exposed to human IFN α, β, and γ, and a mixture of human IFN β plus a twofold excess of rabbit polyclonal antibodies to human IFN β, respectively. (B) The kinetics of IFN-induced changes in diacylglycerol levels in Daudi cells. Cells were exposed to human IFN's α (○) and β (●) for the times indicated. The diacylglycerol level in control cells is indicated by (□) and in cells exposed to a mixture of human IFN β plus a twofold excess of rabbit polyclonal antibodies to human IFN β is indicated by (♦). All diacylglycerol values are mean ± SD of duplicate experiments and IP_2 and IP_3 values are mean ± SD of triplicate experiments. Experimental details are as described (*20*).

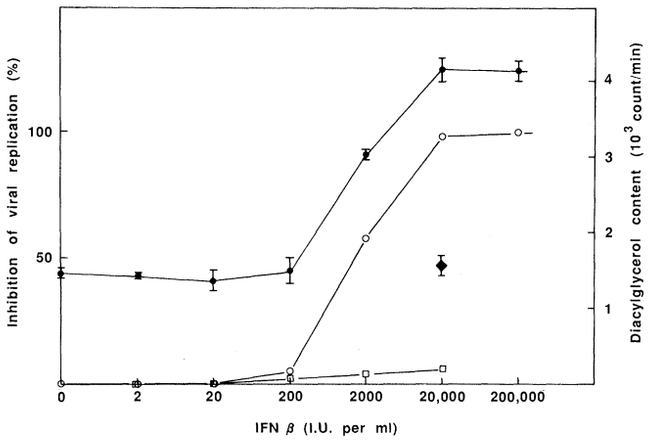

Fig. 2. Correlation between the rise in diacylglycerol level and the antiviral effect of human IFN β in human diploid fibroblasts exposed for 30 seconds to different concentrations of IFN β. Diacylglycerol levels and antiviral state in IFN-treated cells are indicated by (●) and (○), respectively. Diacylglycerol level and antiviral state of human diploid fibroblasts exposed to a mixture of human IFN β (20,000 IU/ml) and fivefold excess of rabbit polyclonal antibodies to human IFN β are indicated by (♦) and (□), respectively. The values for diacylglycerol are mean ± SD of triplicate experiments. Procedures for the induction and assay of the antiviral state are as described (*12*).

phate (Fig. 1A, inset). These increases in inositol phosphates were apparently not accompanied by measurable increases in cytosolic-free Ca^{2+} during the first hour of exposure to IFN β at 20,000 IU/ml. Even so, it is not possible to completely rule out that small and rapid changes in cytosolic-free Ca^{2+} in IFN-treated cells were not detected by the present procedures (*6*).

Mills *et al.* (*13*) reported that IFN's do not signal cells by rapid alterations in phosphatidylinositide levels, which apparently is contradictory to this report. Their measurements were taken 15 minutes after the cells were exposed to IFN. By then, however, as we have shown here, the rapid rise and fall in phosphatidylinositide levels has already transpired.

Several biologically active substances besides IFN are known to stimulate increases in diacylglycerol that may be accompanied by corresponding increases in inositol phosphates (*14*). It is not clear how a single substance like diacylglycerol can act as a second messenger for different biological response modifier molecules. Not all diacylglycerol responses to the binding of various ligands are identical. For example, insulin induces increases in diacylglycerol with no change in the concentrations of inositol phosphates or cytosolic-free Ca^{2+} (*15*). In the case of IFN, diacylglycerol and inositol phosphate concentrations are increased. The kinetics of some of these second messenger responses are also different. For example, insulin, α_1 adrenergic agonists, and platelet-derived growth factor induce relatively slow and sustained increases in diacylglycerol concentration in their target cells (*14, 15*). In contrast, thrombin induces a rapid and transient rise in diacylglycerol in platelets (*16*). The kinetics of IFN-stimulated diacylglycerol and inositol phosphate increases in human cells reported here resemble the effect of thrombin on platelets.

It is possible that these differences produce signals that can activate internal receptors having different turnover rates and affinities with regard to a second messenger yet to be identified. The activated receptor(s) triggers specific metabolic pathways associated with the expression of unique biological activities. Protein kinase C has been reported to be the internal receptor for diacylglycerol (*17*), and it is possible that this enzyme is functioning in this role in IFN-treated cells (*18*). However, the mechanism by which it mediates IFN activity is still unknown. Efforts can now be directed towards the identification and characterization of the postulated internal receptor(s) and its associated metabolic pathway(s) involved in the actions of IFN.

Table 2. Diacylglycerol levels in human fibroblasts containing one, two, and three copies of chromosome 21 exposed to human IFN's α and β. These cells are human fibroblasts derived from skin biopsies. For each additional copy of chromosome 21, the fibroblasts were found to be 4.0 to 5.0 times more sensitive to the antiviral effect of both human IFN's α and β. The cells were grown to a density of 2.0×10^4 cells per square centimeter. They were exposed to different concentrations of IFN for 30 seconds at 37°C and immediately extracted and assayed for diacylglycerol (*14*). Concentrations giving the maximum increases in diacylglycerol levels are shown. Diacylglycerol levels were measured in duplicate experiments. The fold increase is the mean and SD of duplicate experiments; N.D., not determined.

Cell	Karyotype	α		β	
		Concentration (IU/ml)	Fold increase	Concentration (IU/ml)	Fold increase
GM230	45XX-21	24,000	2.73 ± 0.14	24,000	2.60 ± 0.02
GM137	45XY-21	24,000	2.60 ± 0.18	24,000	2.36 ± 0.24
pH 1	46XX	6,000	2.64 ± 0.20	6,000	2.56 ± 0.22
pH 2	46XY	6,000	2.58 ± 0.06	6,000	2.66 ± 0.16
pH 3	46XY	6,000	2.49 ± 0.26	6,000	2.46 ± 0.18
pH 4	46XY		N.D.	6,000	2.50 ± 0.14
GM2504	47XY+21	1,500	2.58 ± 0.10	1,500	2.68 ± 0.12
DSSG-1	47XY+21		N.D.	1,500	2.42 ± 0.06

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20. Human diploid fibroblasts (5×10^6 cells) in 75-cm² plastic flasks or Daudi cells (5×10^6 cells) in sus-

pension culture were prelabeled with [2-^{3}H]-glycerol (6μCi/ml, Amersham, Ontario). The cells were treated with IFN and assayed for diacylglycerol levels as described by Habenicht *et al.* (*14*). Similarly, IFN-treated fibroblasts prelabeled with myo-[2-^{3}H]inositol (10 μCi/ml, Amersham, Ontario) were assayed for inositol phosphate levels according to the method of M. J. Berridge, J. P. Heslop, R. F. Irvine, K. D. Brown, *Biochem. J.* **222**, 195 (1984). IFN's α, β, and γ used in this study were recombinant human IFN's obtained from Triton Biosciences and Dr. W. Berthold. The rabbit polyclonal antibodies to human IFN β were produced in this laboratory. IFN activity was standardized to NIH-WHO-GO2-901-527 (IFN α), NIH-WHO-GO2-902-527 (IFN β), and Gg-901-530 (IFN γ).

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Central Projections of Identified, Unmyelinated (C) Afferent Fibers Innervating Mammalian Skin

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Unmyelinated (C) fibers are the most numerous sensory elements of mammalian peripheral nerve and comprise many of those responsible for initiating pain and temperature reactions; however, direct evidence has been lacking as to where and how these fibers terminate in the central nervous system. A plant lectin (*Phaseolus vulgaris* leukoagglutinin) was applied intracellularly by iontophoresis as an immunocytochemical marker. This permitted visualization of the central terminations of cutaneous C sensory fibers that had been identified by the nature of stimuli that excited them. The central branch of C-fiber units arborized and terminated mainly in the superficial layers of the spinal dorsal horn in defined patterns that related to their functional attributes. Thus, the superficial dorsal horn seems to act as a processing station for signals from fine sensory fibers.

NEURONAL FUNCTION IS DETERmined not only by the characteristics of the individual nerve cell but also by the connections made by the neuron. Therefore, the absence of direct information as to how and where the unmyelinated (C) fibers (the most numerous primary sensory fibers of vertebrates) terminate in the central nervous system has been a hindrance to understanding neural arrangements for the sensory systems of mammals.

The central terminations of myelinated fibers of particular functions have been established by marking individual fibers intracellularly with the tracer substance horseradish peroxidase (HRP) after determining the nature of the afferent messages of the fiber during electrophysiological recordings (*1*). Unmyelinated fibers are extremely fine (less than 1 μm in diameter), and stable intracellular recordings of and intracellular injections into such small fibers have not been possible. An alternative, injecting the larger cell bodies of these neurons in the dorsal root ganglia (DRG), has been problematic because the distance from the spinal cord appears too great for orthograde HRP transport in the very fine fibers (*2*). To circumvent the transport problems we have used a plant lectin, *Phaseolus vulgaris* leukoagglutinin (PHA-L), which readily fills distant processes by orthograde transport (*3*) and can be iontophoresed into cell bodies from micropipette electrodes.

A small rodent (guinea pigs, 150 to 300 g) was used for these experiments to minimize the transport distance between the DRG and the spinal cord. Under surgically clean conditions and deep pentobarbital anesthesia, a laminectomy and dissection exposed either the cervical 2 (C2) or the lumbar 6 (L6) ganglion and a major nerve supplying it—the great occipital nerve for C2 and the sciatic nerve for L6. The nerve was stimulated electrically (once every 2 to 3 seconds) with brief pulses at an intensity sufficient to excite C fibers to establish the afferent fiber's conduction velocity. Fine micropipettes (less than 0.2 μm in diameter at the tip) containing a 2.5% solution of PHA-L dissolved in 0.1*M* phosphate buffer (*p*H 7.4) were used to record intracellularly from the DRG cell bodies. When a recording was obtained from the C-fiber neuron (conduction velocity, 0.3 to 1 m per second), the receptive field of the nerve was explored with a systematic progression of "natural" stimuli—static and moving contact of the skin, skin cooling, radiant heat, mechanical pressure, and noxious mechanical stimulation (*4*). We were only able to classify neurons with cutaneous or immediately subcutaneous receptive fields. Previously established criteria were used to identify each type of C-fiber sensory unit (*5*).

After determining the nature of the stimuli that effectively excited the unit, PHA-L was deposited intracellularly by positive iontophoretic current (10×10^{-9} A for 2 minutes or more). One cell was labeled in each animal as verified by determining that PHA-L appeared in only one DRG cell body. The wounds were closed with standard surgical techniques. An antibiotic was administered and the animal's general condition was monitored while it recovered from anesthesia and for 2 to 7 days afterwards. (Surviving

Table 1. Terminal domains of each type of C fiber. The cells stained in the C2 ganglion were immunohistologically processed after 2 days of survival, and those in the L6 ganglion after 5 to 6 days of survival. The extension of each terminal area (the dense areas of enlargements and terminals in Figs. 1 and 2) in the histological sections was measured with an ocular micrometer or calculated by a computer three-dimensional graphics program. The results with these seven fibers were consistent with observations on less complete arborizations in other similar units stained with PHA-L or HRP. The extent and distribution of the central branches of a fiber were greater than these terminal domains. Laminae of the terminal area were classified according to Rexed (*12*) and Light and Perl (*1*).

Sensory modality of receptive field	Segmental level (ganglion)	Conduction velocity (m/sec)	Terminal area			
			Rostrocaudal extension (μm)	Dorsoventral extension (μm)	Mediolateral extension (μm)	Laminae
High-threshold mechanoreceptor	C2	0.5	280	100	150	II_o, part of II_i
Polymodal nociceptor	C2	0.5	300	50	200	II_i
	L6	0.5	600	150	200	I, II_o, III, IV
Mechanical cold nociceptor	C2	0.5	400	300	150	I, part of II_o
	L6	0.5	450	100	150	I, part of II_o
Low-threshold mechanoreceptor	C2	0.5	380	50	120	II_o, part of II_i
	C2	0.6	300	50	100	II_i, II_o

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