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How cells respond to interferons revisited: From early history to current complexity

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Abstract

A brief account of how I became involved in interferon research is followed by recollections of key experiments that led to the discovery of the roles of the JAKs and STATs in interferon-dependent signaling. I then outline the complex responses of cells to interferons, including the roles of kinases other than JAKs and transcription factors other than STATs, differential responses to interferons α and β , modulation of response by prior exposure to other cytokines (“priming”), cytokine-dependent induction of high level expression of STATs 1 and 3 and the activation of a new set of genes by these unphosphorylated STATs, and diverse patterns of STAT activation in different cell types in response to a single interferon.

Introduction

It is indeed an honor to have this chance to record my appreciation of the Milstein family and to recognize their unflagging support of interferon research over many years. I received the Milstein Award of the International Society for Interferon and Cytokine Research in 1997, together with Ian Kerr and Jim Darnell, rewarding the work of all three labs that led to the discovery of the JAKs and STATs, the kinases and transcription factors that power responses to interferons. The initial discovery was quickly followed by the recognition that the JAKs and STATs are essential for signaling in response to most cytokines and play additional roles in biology that are still being uncovered. There is still much work to do to understand the full complexity of how cells respond to interferons.

My life in science, not the restaurant business

This is the title of a “Reflection”, published recently in the *Journal of Biological Chemistry* [1]. You can find out almost everything worth knowing about me by reading it. I started my scientific career as a protein chemist and continued in this field as an Assistant Professor of Biochemistry at Stanford University. I began to learn about animal cells and viruses during a sabbatical year with Lionel Crawford at the Imperial Cancer Research Fund in London in 1970-71. For my second sabbatical, in 1977-78, I persuaded my old friend Ian Kerr to let me spend a year in his laboratory at the National Institute for Medical Research at Mill Hill in London. I was fascinated by Ian's marvelous discovery that 2', 5'-oligoadenylates, formed in interferon-treated, virus-infected cells, were a key intermediate in the cellular defense against infection, leading to degradation of viral and cellular RNAs by activating the interferon-induced ribonuclease L. For a short time thereafter, I pursued in my laboratory at Stanford the

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hypothesis that a series of related compounds, also formed by 2', 5'-oligoadenylate synthetase when it is activated by double-stranded RNA, had significant alternative roles in biology, a good idea whose time has not yet come.

Interferon-induced genes and factors

Continuing discussion and collaboration with Ian led to our decision to begin investigating how the binding of interferons to their receptors led to the activation of gene expression. A key early paper, from the work of my graduate student Richard Friedman, also included work done in Ian's lab by his student Martin McMahon [2]. We treated human neuroblastoma cells with type I interferon, constructed a cDNA library in a phage λ vector from the mRNAs present in the treated cells, and screened the library differentially with probes made from the RNAs of treated and untreated cells. Eighteen cDNAs were isolated, corresponding to seven different mRNAs that were induced up to 40-fold by interferon. The 6-16, 9-27 and 1-8 cDNAs, named for the plate and clone numbers assigned during screening, correspond to strongly induced mRNAs of unknown function and were extremely valuable in later experiments. In a continuation of his thesis work, Richard examined the promoters of several previously known genes that were also induced by type I interferons, namely, metallothionein-II and three HLA genes [3], revealing homologous sequences that were shown to be interferon-stimulated regulatory elements (ISREs) in later functional experiments. At about this time, in 1983, I moved to the Imperial Cancer Research Fund in London to facilitate the productive collaboration with Ian. Trevor Dale, a graduate student in the London laboratory, contributed an important functional study of the ISREs, showing that the conserved element of the 6-16 gene conferred the ability to be induced by interferon to a heterologous promoter and that the element was bound to an interferon-induced protein complex [4]. Subsequent work that comprised an additional part of Trevor's thesis showed that this interferon-induced "E factor" was formed in less than a minute in cytoplasmic extracts of treated cells and even in extracts of enucleated cells, but that it quickly became bound to DNA [5]. We formulated the following hypothesis: "...these results suggest a model for signal transduction in which latent E factor, located in the cytoplasm, is activated or released from an inhibitor very rapidly upon binding of IFN- α to its receptor. Active E factor can then migrate to the nucleus, where it binds to the IFN-stimulated regulatory elements of IFN-regulated genes, activating their transcription" [5]. Since the current article is intended to be a personal account, I have recalled only the history of our own work, but of course related experiments were being carried out in other laboratories, especially the laboratory of Jim Darnell at Rockefeller University. Please see reviews [6,7] for descriptions and results of these experiments.

From descriptive experiments to specific signaling components

At this point, it was clear that the next important step was to identify the specific proteins that comprised the signaling pathway. Jim Darnell's laboratory concentrated on biochemical purification of the complex that we had dubbed "E factor", identified independently and named ISGF-3, leading to the cloning of its components, STAT1, STAT2 and IRF9 (alias p48) [8,9]. We favored a genetic approach, selecting mutant cell lines defective in responding to interferons that could be complemented by expression of the protein that had been ablated by chemical mutagenesis [10]. Fortunately, the two quite different methodologies turned out to be highly complementary, facilitating rapid delineation of the details of signaling, as described in reviews [6,7]. The first mutant (initially called 11, 1 and later U1A) was very difficult to obtain because we did not yet appreciate the extensive mutagenesis that was needed in order to knock out both alleles of a gene and thus to obtain a recessive mutant lacking expression of some protein required for signaling [10]. However, by dint of hard work and amazing insight and persistence, Sandra Pellegrini, who isolated 11, 1 originally in London, managed to complement this mutant in her own laboratory in Paris and thus to discover that Tyk2, a member

of the JAK family of kinases, was required for cells to respond to type I interferons [11]. When we appreciated that hitting the cells harder with a chemical mutagen made the isolation of mutants much easier [12], we were soon able to accumulate a collection of eight mutant cell lines defective in response to either type I interferon, type II interferon, or both. Each of these lines was shown to lack a single specific protein required for signaling. Here is a list of the mutant cell lines and the proteins in each whose expression was affected by mutation: U1A, Tyk2; U2A, IRF9; U3A, STAT1; U4A, JAK1; U5A, IFNAR2; U6A, STAT2; γ 1A, JAK2; γ 2A, IFNGR1. Tyk2, IRF9, STAT2 and IFNAR2 are required only for type I interferons and JAK2 and IFNGR1 are required only for type II interferon. JAK1 and STAT1 are required for both types of interferon, and so the mutant cell lines lacking these proteins are doubly defective. Since the JAKs and STATs function in many different signaling pathways, especially those stimulated by cytokines, the mutant cell lines cleanly lacking expression of single JAKs or STATs have been valuable in many different studies, and we have been happy to provide these cells to more than 400 different investigators to date! With the success of generating a set of mutants in interferon-dependent signaling pathways under our belts, we undertook additional studies to develop more convenient methods, since functional complementation of the mutants obtained after chemical mutagenesis proved to be difficult—with the exception of U1A, the protein missing in each of the other seven cell lines was identified by complementation with a cDNA clone that had been obtained by other means. Our new approach utilizes insertion of promoters into the genome, using retroviral or lentiviral vectors or transposable elements, to generate dominant mutants in which enhanced or inappropriate expression of a gene or fragment of a gene gives a phenotype that can be selected. The approach is described in detail in recent reviews [13,14] and has been applied to an analysis of the activation of NF κ B [15]. We are also pursuing related methods to generate recessive mutants in which one allele of a gene whose product is required for signaling is marked by insertion and the other is mutated or deleted.

The combination of cDNAs encoding STATs 1 and 2 from the Darnell laboratory and our mutant cell lines lacking expression of these two proteins provided the basis for many collaborative mechanistic studies. In an early and important example, Shuai *et al.* [16] showed that the phosphorylation of a single tyrosine residue of STAT1 is required for gene activation in response to IFN- γ by expressing mutant versions of the protein in U3A cells. Many additional experiments along similar lines are described in the two comprehensive reviews cited [6,7].

Current complexity

No biological phenomenon is as simple as it appears when first discovered, and interferon-dependent signaling is certainly no exception. Looking back, it is hard to believe now how naïve I was in the early days in thinking that everything needed for interferons to activate gene expression had been revealed by the discovery of the receptors, the JAKs and the STATs. As we and others continued to dissect the pathways, it became apparent that many more components were needed to account for the complexity of how cells respond to interferons. Below, I discuss briefly several aspects of this complexity, which are covered in much greater depth in two recent reviews dealing separately with type I [17] and type II [18] interferons.

Activation of additional kinases and transcription factors

As listed in the Figure, several different kinases and transcription factors are activated by interferons, but almost certainly not all in every type of cell. It is likely that the kinases connect to the transcription factors, but in ways that are not yet well understood. For example, for its efficient function in transcription, STAT1 must be phosphorylated on serine 727 as well as on tyrosine 701, but how the serine phosphorylation is achieved in a variety of different cells is still being worked out. Additional transcription factors that are activated by interferons may

act independently of STATs to drive the expression of certain genes, or may collaborate with activated STATs, as shown in the Figure. An illustration of the latter possibility is provided by our work showing that, in order to respond to IFN- γ , some genes need activation of the NF κ B subunit p65 in response to interferon-dependent activation of I κ B kinase β in a pathway that is distinct from the liberation of NF κ B from its inhibitor I κ B [19 and unpublished data of David Shultz and GRS]. It is interesting that IFN- β has effects that are distinct from those of IFN- α subtypes, exemplified by its differential ability to activate expression of the *CXCL11* and *TRAIL* genes, which depends on its differential ability to activate PI3 kinase and to phosphorylate NF κ B [reviewed in 17].

Priming and functions of unphosphorylated STATs

How cells respond to interferons is conditioned by their prior exposure to cytokines, a complex phenomenon called priming that has been reviewed recently in detail [17,18]. An important component of priming is the ability of activated STAT1 to drive expression of the *STAT1* gene, leading to a large (ca. 20-fold) increase in the concentration of unphosphorylated STAT1 in IFN- γ -treated cells several hours after the initial contact with interferon. Similarly, the *STAT3* gene has a GAS element that drives its expression in response to the activation of STAT3, for example, in response to IL-6 [19]. As discussed below, the IL-6 and IFN- γ receptors preferentially activate STAT3 and STAT1, respectively, but also activate the other STAT with lower efficiency. The ratio of STAT3 to STAT1 may be as much as 400 times higher in cells pretreated with IL-6 than in cells pretreated with IFN- γ , with significant consequences for downstream signaling in response to a second cytokine that activates either STAT, for example, IFN- β . Perhaps a more important consequence of upregulated STAT expression is the ability of unphosphorylated STAT1 and STAT3 to drive gene expression through mechanisms distinct from those used by phosphorylated STATs. Aseem Kumar uncovered this phenomenon by showing that STAT1-null U3A cells were defective in TNF-induced apoptosis due to low expression of caspases and that this defect could be corrected not only by wild-type STAT1 but also by the Y701F mutant of STAT1, which cannot be phosphorylated on tyrosine [20]. Although we have done some work on gene expression due to unphosphorylated STAT1 [reviewed in 21], currently we have a much better understanding of the function and importance of unphosphorylated STAT3, which drives the expression of many genes, including several that are clearly involved in tumorigenesis [22]. Recent work has shown that unphosphorylated STAT3 accumulates in IL-6-treated cells long after the level of phosphorylated STAT3 has declined due to the activity of SOCS3 and other negative regulators. Unphosphorylated STAT3 then forms a complex with NF κ B, in competition with I κ B. The nuclear localization signal of STAT3 drives this complex into the nucleus, where it binds to the κ B elements of the regulated genes to activate their expression. The genes so regulated are a small subset of those that respond to TNF α through conventional activation of NF κ B (Jinbo Yang and GRS, unpublished).

Differential activation of STATs by interferons in different cell types

When STAT1 is absent, IFN- γ activates STAT3 robustly and, conversely, when STAT3 is absent, IL-6 (and presumably other gp130-linked cytokines) activates STAT1 robustly (reviewed by Ramana, *et al.* [23]). It is interesting that, in addition to JAK1 and JAK2, SRC-family kinases are required to activate STAT3 in response to IFN- γ [24]. Because STAT1 and STAT3 usually have opposite biological effects, their reciprocal activation in response to IFN- γ or IL-6 suggests that their relative abundance, which may vary considerably in different normal cell types, under different conditions, or in tumors, may well have a major impact on how cells behave in response to these two different classes of cytokines [24]. Even when no STAT is missing, type I interferons can activate all seven of the STATs, to varying extents in different cell types (reviewed by van Boxel-Dezaire *et al.* [17]). In recent experiments, we have

observed that, in freshly drawn human blood treated for 30-45 min with IFN- β , STATs 1, 3 and 5 are activated to very different extents in CD4⁺ and CD8⁺ T cells, B cells and monocytes, with little activation of STAT1 in CD4⁺ T cells and B cells (Anette van Boxel-Dezaire and GRS, unpublished)! It seems inevitable, therefore, that the interferon-induced patterns of gene expression will be very different in these different cell types, as will the biological consequences of exposure to interferon. It also seems likely that these results represent the tip of the iceberg; to fully understand the biological effects of interferons, we scientists are likely to have to investigate signaling and downstream events in many different cell types. Good employment for the future!

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Biography

George R. Stark, Ph.D.

George Stark has contributed to several different areas of science. Early work on enzyme mechanisms and protein chemistry led to the development of the Northern and Western techniques for analysis of specific RNAs and proteins. His laboratory has also studied gene amplification in mammalian cells, leading to an appreciation both of the mechanisms that generate amplified structures and the regulatory processes that prevent amplification from occurring in normal cells. A major project was to apply systematic genetic analysis to interferon-dependent signaling pathways. In collaboration with Dr. Ian Kerr, eight different mutant cell lines were isolated, each complemented by a cDNA encoding a different protein in the pathway. The work with interferon, together with that of Dr. James Darnell, led to the discovery of the family of JAK-STAT signaling pathways, which mediate responses to many different extracellular factors. A similar genetic approach is now being used to analyze signaling pathways that activate NF κ B, to isolate and characterize negative regulators of NF κ B, and to identify novel aspects of p53-dependent signaling.

Dr. Stark was born in New York City in 1933 and earned a Ph.D. degree in Chemistry from Columbia University in 1959. After a postdoctoral fellowship with Drs. William Stein and Stanford Moore at the Rockefeller University, he joined the Department of Biochemistry at Stanford University in 1963, becoming Professor in 1971. In 1983, he moved to the Imperial Cancer Research Fund in London as Associate Director of Research. In July 1992, he became the Chair of the Lerner Research Institute of The Cleveland Clinic Foundation, a position he held until August 2002. In October 2002, he was elected to the Institute of Medicine of the National Academy of Sciences. He is currently the Distinguished Scientist of The Cleveland Clinic Foundation, with a laboratory in the Department of Molecular Genetics, and a Professor of Genetics at Case Western Reserve University. Dr. Stark was elected to the National Academy of Sciences in 1986, to the Fellowship of the Royal Society in 1990 and to the Institute of Medicine in 2002. He has also received the Sober, Milstein and Coley Awards.

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Figure.

Complexity of responses to interferons. In addition to the activation of JAKs and STATs, interferons activate other kinases and transcription factors (TFs), as indicated in detail in the figure. The patterns of STAT, TF and kinase activation in response to each specific interferon are likely to vary substantially among different cell types. Some genes respond only to activated STATs, some only to activated TFs and some only to both together. Examples are given in the figure. For more details, see the reviews [17,18].