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# Purification and Cloning of Interferon Alpha

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**Abstract** Interferon alpha (IFN-α) was approved by the US Food and Drug Administration on June 5, 1986 and paved the way for development of many other cytokines and growth factors. Nevertheless, we have barely touched the surface of understanding the multitude of human IFNs. This paper reviews the history of the purification of human leukocyte IFN, the cloning of the IFN-αs, and the current state of knowledge of human interferon alpha genes and proteins.

## 1 The Interferons

The interferons are proteins with antiviral activity (Isaacs et al. 1957; Isaacs and Lindenmann 1957; Nagano and Kojima 1958; Pestka et al. 1987, 2004a; Sen and Lengyel 1992; Stark et al. 1998). There are two types of human interferons, type I and type II, and interferon-like cytokines (Krause and Pestka

**Table 1** The human type I interferons and interferon–like proteins[a]

| Class | Names |
|---|---|
| Type I IFNs | IFN–α |
| | IFN–β |
| | IFN–ε |
| | IFN–κ |
| | IFN–ω |
| | IFN–ν |
| Interferon–like cytokines | IL–28A, IL–28B, IL–29 |

[a]This table summarizes interferon and interferon-like proteins. Human type I IFN members are clustered together, as are the IL-28A, IL-28B, and IL-29 cytokines

2005; Pestka et al. 2004a, 2004b). Type I human interferons consist of six classes: IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, and IFN-ν (Table 1). However, a large number of type I interferons are found in other animal species, which will not be discussed in this report, but they have been described in another publication (Krause and Pestka 2005). Type II interferon consists only of IFN-γ. In addition, three related human interferon-like cytokines have been reported: IL-28A, IL-28B, and IL-29 (Table 1) (Kotenko et al. 2003; Sheppard et al. 2003). There is only one IFN-β, IFN-ε, IFN-κ, IFN-ω, and IFN-ν, but the IFN-α proteins consist of twelve individual proteins (Table 2).

Although partial purification of the interferons as bands on sodium dodecyl sulfate-polyacrylamide gels was reported by a number of groups, it was not until 1978 and thereafter that any interferon had been purified to homogeneity in solution in sufficient amounts for its chemical and physical characterization (Friesen et al. 1981; Rubinstein et al. 1978a, 1979c, 1981; Stein et al. 1980). The introduction of reverse-phase and normal-phase high-performance liquid chromatography to the purification of proteins (Friesen et al. 1981; Rubinstein et al. 1979c; Stein et al. 1980) led to the first successful purification of IFN-α and IFN-β so that sufficient amounts were available in solution without detergent for their chemical, biological, and immunological studies.

## 2 Purification of Human Leukocyte Interferon

Since the discovery of interferons, many attempts were made to purify the interferons with little success until 1978. In fact, interferon used in experiments as well as in initial human clinical trials was essentially a crude protein fraction less

**Table 2** Human interferon alpha genes (14) and proteins (12/13)[a]

| Genes | Proteins |
|---|---|
| IFNA1 | IFN–αD, IFN–α1 |
| IFNA2 | IFN–αA (IFN–α2a), IFN–α2 (IFN–α2b), IFN–α2c |
| IFNA4 | IFN–α4a (IFN–α76), IFN–α4b |
| IFNA5 | IFN–αG, IFN–α5, IFN–α61 |
| IFNA6 | IFN–αK, IFN–α6, IFN–α54 |
| IFNA7 | IFN–αJ, IFN–αJ1, IFN–α7 |
| IFNA8 | IFN–αB2, IFN–αB, IFN–α8 |
| IFNA10 | IFN–αC, ΨIFN–α10, ΨIFN–αL, IFN–α6L |
| IFNA13 | IFN–α13 (sequence identical to IFN–α1) |
| IFNA14 | IFN–αH, IFN–αH1, IFN–α14 |
| IFNA16 | IFN–αWA, IFN–α16, IFN–αO |
| IFNA17 | IFN–αI, IFN–α17, IFN–α88 |
| IFNA21 | IFN–αF, IFN–α21 |
| IFNAP22 | ΨIFN–αE |

[a]The genes for human IFN–α are given in the left column and the proteins in the right column. Allelic forms of the IFN–α proteins are shown in the right column (Pestka 2000). Designations in parentheses represent alternate names for identical proteins

than 1% of which by weight consisted of interferon. Because of the use of such crude interferon-containing material, it was not clear what activities of these preparations were indeed due inherently to the interferon present and what activities were due to the numerous other contaminating proteins. By definition, the antiviral activity was due to the interferon. However, these crude preparations exhibited antiprotozoal and antibacterial activities, inhibited cellular growth (antiproliferative activity), blocked antibody synthesis, and were ascribed to have many other activities. However, without high-purity preparations of interferon, it was not possible to demonstrate definitively whether or not a particular activity was due to the interferon protein molecule itself. Thus, it was essential to obtain purified interferon to determine what activities were intrinsic to the interferon molecule. Since very little was known about the size and structure of the interferons, the isolation of purified interferons was necessary to establish their chemical composition and structure as well as their biological activities.

## 2.1 Production

We began purification of interferon from human leukocytes in 1977. This human leukocyte interferon (Hu-IFN-α) was produced by incubating human white blood cells with Newcastle disease virus or Sendai virus for 6–24 h (Familletti et al. 1981a; Familletti and Pestka 1981; Hershberg et al. 1981; Waldman et al. 1981). The procedure was a combination of methods that had previously been reported (Cantell and Tovell 1971; Wheelock 1966). The antiviral activity was found in the cell culture medium after overnight incubation of the leukocytes. We substituted milk casein for human or bovine serum in the culture medium, as had been described (Cantell and Tovell 1971). The use of casein, a single protein, instead of serum, which contains many different and uncharacterized proteins, simplified the initial concentration and purification steps. We used leukocytes from normal donors as well as from patients with chronic myelogenous leukemia. These leukemic cells made substantial amounts of human leukocyte interferon when induced with Newcastle disease virus or Sendai virus (Familletti et al. 1981a; Hadhazy et al. 1967; Lee et al. 1969; Rubinstein et al. 1979b); however, less than 0.1% of the starting medium consisted of leukocyte interferon (Rubinstein et al. 1981). Although leukocyte interferon consisted predominantly of IFN-α, small amounts of IFN-β and IFN-ω were also present (Adolf et al. 1990; Cavalieri et al. 1977b).

The cytopathic-effect inhibition assay for interferon as originally described took 3 days. Other assays for interferon were even longer. A more rapid assay was necessary to proceed with the purification expeditiously. A cytopathic effect inhibition assay that could be done in 12–16 h was developed and accelerated the purification immensely (Familletti et al. 1981b).

## 2.2 High-Performance Liquid Chromatography for Protein Purification

Prior to purification by high-performance liquid chromatography (HPLC), interferon in media after production was concentrated, then passed through a Sephadex G-100 fine gel filtration column to isolate protein in the 20,000-mw range as described (Rubinstein et al. 1978a, 1979c, 1981). Because standard methods for protein purification were not significantly successful in purification of the human interferons from blood cells, we applied HPLC to the purification of proteins. Udenfriend and co-workers (Bohlen et al. 1975; Stein et al. 1973; Udenfriend et al. 1972) had developed sensitive fluorescent techniques for detection of amino acids and peptides and had achieved the separation of peptides by reverse-phase HPLC (Lewis et al. 1978; Rubinstein et al. 1978b; Stein

et al. 1978), but separation of proteins had not yet been accomplished. After gel filtration, the major interferon fraction was applied directly to a Lichrosorb RP-8 column. The column was washed in 1 M sodium acetate buffer, then the interferon was eluted with *n*-propanol gradients, as reported and described in detail (Rubinstein et al. 1978a, 1979c, 1981). By changing the pH of the elution buffer, a completely different separation could be achieved during elution of the same reverse-phase column with *n*-propanol. As subsequently demonstrated with fibroblast interferon (Friesen et al. 1981), a large number of different columns and solvent systems could be used to effect resolution of proteins. By applying normal-phase chromatography with a diol silica column between the two reverse phase columns, it was possible to use just three sequential HPLC steps to purify human leukocyte interferon (IFN-α) to homogeneity. Sufficient amounts were purified in high yield for initial chemical characterization of the protein and for determination of amino acid composition. The amino acid composition of the human leukocyte interferon species γ2 (our nomenclature at the time for what is now designated as one of the IFN-α species) was the first reported for any purified interferon (Rubinstein et al. 1979c). Originally, the natural interferons that were isolated from the mixture present in leukocyte interferon by high performance liquid chromatography (Pestka 1983a; Rubinstein et al. 1978a, 1979c, 1981) were then designated α1, α2, β1, β2, β3, γ1, γ2, γ3, γ4, γ5, and δ. Unfortunately, the same Greek letters were later used to designate leukocyte, fibroblast, and immune interferons, respectively, as IFN-α, IFN-β, and IFN-γ, causing a great deal of misunderstanding for many years.

The initial steps for purification of IFN included selective precipitations and gel filtration (Fig. 1) followed by HPLC. The HPLC steps were reverse-phase chromatography (Fig. 2A) at pH 7.5 on LiChrosorb RP-8, normal partition chromatography on LiChrosorb Diol (Fig. 2B), and reverse-phase chromatography at pH 4.0 on LiChrosorb RP-8 (Fig. 2C, D). Gradients of *n*-propanol were used for elution of interferon from these columns (Fig. 2A, C, D). The overall purification was approximately 80,000-fold and the specific activity of purified interferon was $2–4\times10^8$ units/mg (Rubinstein et al. 1979c). Interferon prepared by this procedure yielded a single band of MW 17,500 on polyacrylamide gel electrophoresis. The antiviral activity was associated with the single protein band (Rubinstein et al. 1978a). The specific activity of this peak was $4\times10^8$ units/mg.

Several reports had previously described high-performance liquid chromatography of proteins, mainly on ion exchange and size exclusion columns (Chang et al. 1976; Regnier and Noel 1976). However, those systems were either not commercially available or had a low capacity. With proper choice of eluent and pore size, octyl and octadecyl silica could be used for high-resolution reverse-phase HPLC of both peptides and proteins. Accordingly, with *n*-propanol

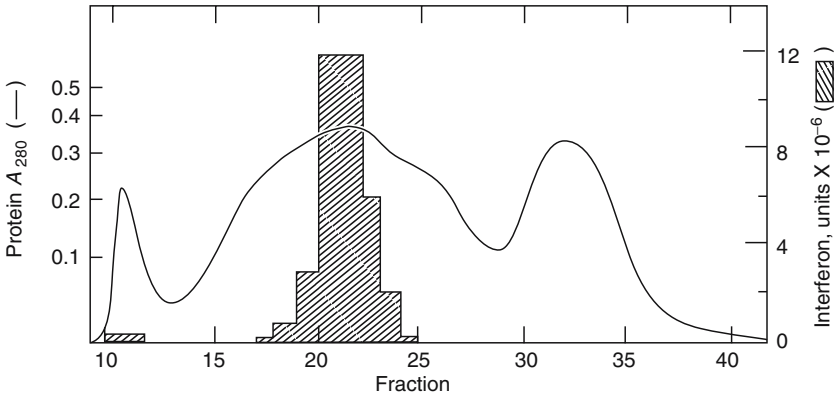


**Fig. 1** Initial steps in purification of leukocyte interferon. Cells and debris were removed by low-speed centrifugation from the medium containing interferon. Casein was used as a serum substitute. By acidification of the medium to pH 4 with hydrochloric acid, the bulk of the casein was precipitated. The supernatant containing the interferon was concentrated as described (Rubinstein et al. 1979c). The concentrated solution containing relatively crude interferon was applied to a column of Sephadex G-100 fine, then the column eluted with 4 M urea/0.1 sodium acetate, pH 7.5. The detailed procedures were described previously (Rubinstein et al. 1979c). The fractions with interferon activity were pooled and purified by HPLC (Fig. 2). Similar procedures were carried out as we purified leukocyte interferon species over several years (Hershberg et al. 1981; Pestka 1983a; Rubinstein et al. 1979c, 1981; Waldman et al. 1981). (From Rubinstein et al. 1979c)

as eluent, the use of LiChrosorb RP-8 (octyl silica) columns for protein fractionation was a major factor in the success of the purification (Fig. 2A, C, D). In addition, LiChrosorb Diol, which is chemically similar to glycophase resins, which have been used for exclusion chromatography of proteins, was introduced as a support for normal partition chromatography of proteins (Fig. 2B). High recoveries of interferon activity were obtained in each chromatographic step, a requirement when small amounts of initial starting material are present. Although the initial experiments were conducted with leukocytes from normal donors (Rubinstein et al. 1978a, 1979c), it was found that leukocytes from patients with chronic myelogenous leukemia (CML), who were undergoing leukapheresis to lower their peripheral white blood cell counts, were a rich source of interferon that appeared to be essentially identical to the human leukocyte interferon purified from leukocytes from normal donors (Rubinstein et al. 1979a). As with HPLC of interferon from normal leukocytes on the Diol column (Fig. 2B), three major peaks of activity, labeled $\alpha$, $\beta$, $\gamma$, were observed with

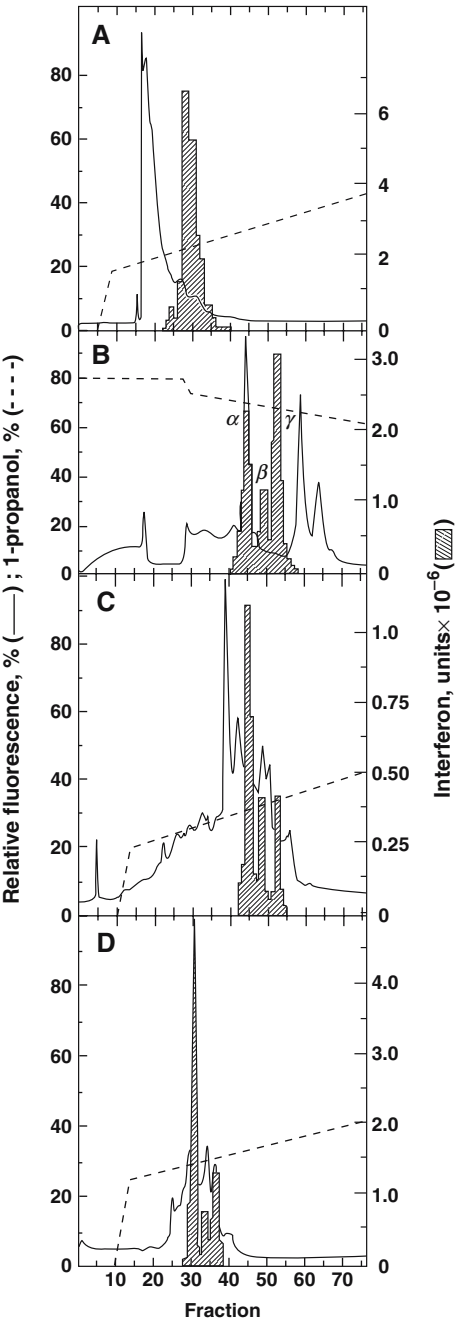


**Fig. 2A–D** High-performance liquid chromatography of leukocyte interferon. **A** Chromatography on Lichrosorb RP-8 at pH 7.5. **B** Chromatography on Lichrosorb diol at pH 7.5. **C** Chromatography on Lichrosorb RP-8 at pH 4.0. **D** Rechromatography on Lichrosorb RP-8. The conditions were similar to those of step **C**. Several preparations carried through step **C** were pooled ($13\times10^6$ units) and applied to the last column. The gradations on the abscissa correspond to the end of the fractions. Further details were reported previously (Rubinstein et al. 1979c). (From Rubinstein et al. 1979c)

interferon prepared from CML cells. Although the protein profiles were almost identical, the activity profiles showed that the amount of activity under peak α was lower in preparations from leukemic cells compared to normal leukocytes (Rubinstein et al. 1979a, 1979c). However, even from normal leukocytes, the ratio of peaks α, β and γ varied from one preparation to another.

### 2.3 Multiple Species of Leukocyte Interferon

During the purification of leukocyte interferon, it became evident that multiple species existed. Peptide mapping and sequencing of these species supported this concept. As additional leukocyte interferon species were isolated from cultured myeloblasts (Hobbs et al. 1981; Hobbs and Pestka 1982) and other sources (Allen and Fantes 1980; Berg and Heron 1981; Zoon 1981), the concept that IFN-αs are a family of interferons was established.

### 2.4 Carbohydrate Content

Five purified species of leukocyte interferon that were initially isolated and interferon produced by Namalwa cells were found to contain no detectable carbohydrate (Allen and Fantes 1980; Rubinstein et al. 1981). A more extensive analysis of the carbohydrate content of the species of human IFN-α subtypes derived from patients with chronic myelogenous leukemia (CML) and from normal donors was determined (Adolf et al. 1991; Labdon et al. 1984). Amino sugar content was measured by HPLC and fluorescamine detection of acid hydrolysates of each sample (Labdon et al. 1984). *O*-linked glycosylation was also detected by a combination of HPLC, enzymatic analysis, and SDS-polyacrylamide gel electrophoresis (Adolf et al. 1991). Two species showed significant amounts of glucosamine (Labdon et al. 1984). Most of the purified species of leukocyte interferon from a myeloblast cell line were also tested and two species were found to contain sugar residues. These forms also differed from the CML interferons in that they revealed the presence of greater amounts of galactosamine. The apparent lack of carbohydrate in some of the higher-molecular-weight species of interferon implicated factors other than glycosylation for the molecular-weight differences. The results indicated that some species of IFN-α are glycosylated to various degrees. It was later shown that a natural form of Hu-IFN-α2 was *O*-glycosylated (Adolf et al. 1991) and that Hu-IFN-ω is glycosylated (Adolf et al. 1990). Considering that the recombinant human IFN-α species produced in *Escherichia coli* do not contain carbohydrate, it was useful to discover that most of the human IFN-α species were devoid of carbohydrate.

## 3
## Identification and Cloning of the Recombinant Human IFN-α Species

Because recombinant DNA technology offered an opportunity to produce large amounts of Hu-IFNs economically, many scientific teams set out to clone them in bacteria. Several groups isolated recombinants for several Hu-IFN-α species (Maeda et al. 1980; Nagata et al. 1980) and for IFN-β (Derynck et al. 1980; Goeddel et al. 1980a; Houghton et al. 1980; Maeda et al. 1980; Taniguchi et al. 1980), obtaining the clones by somewhat different but analogous approaches. The cloning and expression of Hu-IFN-αA (Hu-IFN-α2a) as an illustration of these procedures is described.

Isolating Hu-IFN DNA sequences was a formidable task since it meant preparing DNA recombinants from cellular mRNA that was present at a low level. This task had never been accomplished previously from a protein whose structure was unknown. In addition, in order to reconstruct DNA recombinants which would express natural IFN, it is useful to know the partial amino acid sequence of the proteins, particularly at the $NH_2$- and COOH-terminal ends. Without this information, synthesis of natural Hu-IFN in bacterial cells would not have been definitive. Thus, purification of the Hu-IFNs and determination of their structure (Allen and Fantes 1980; Hobbs et al. 1981; Hobbs and Pestka 1982; Knight et al. 1980; Levy et al. 1981; Rubinstein et al. 1978a, 1979c, 1981; Shively et al. 1982; Zoon et al. 1979) assisted us in these efforts.

To isolate recombinants containing the human DNA corresponding to IFN-α, we developed a number of procedures. First, it was necessary to isolate and measure the IFN mRNA. This was accomplished several years earlier when IFN mRNA was translated in cell-free extracts (Pestka et al. 1975; Thang et al. 1975) and in frog oocytes (Cavalieri et al. 1977a; Cavalieri et al. 1977b; Cavalieri and Pestka 1977; Reynolds et al. 1975). The next step was to prepare sufficient mRNA from cells synthesizing IFN, and this was accomplished with both fibroblasts and leukocytes (Familletti et al. 1981a; McCandliss et al. 1981a). A library of complementary DNA (cDNA) was prepared from a template of partially purified mRNA isolated from human leukocytes synthesizing IFN. Next was to find in this vast library of recombinant plasmids those which contained DNA encoding IFN. We devised an indirect two-stage procedure to identify clones containing interferon sequences. In the first stage, we screened all the bacterial colonies to find those with cDNA made from the RNA of induced cells; among these there might have been some carrying IFN cDNA. We therefore screened all the recombinants for their ability to bind to mRNA from cells synthesizing IFN (induced cells), but not to mRNA from uninduced cells (those not producing IFN). To do this, individual transformed colonies were screened by colony

hybridization for the presence of induced specific sequences with $^{32}$P-labeled IFN mRNA (mRNA from induced cells) as probe. In the presence of excess mRNA from uninduced cells, recombinants that were representative of mRNA sequences existing only in induced cells should be evident on hybridization. This screening procedure allowed us to discard about 90% of the colonies: since their plasmids carried no induced cDNA, these could not encode IFN (Maeda et al. 1980, 1981).

In the second stage, we identified those recombinants containing the IFN DNA sequences among the remaining 10%. To accomplish this, we pooled the recombinant plasmids in groups of ten and examined these for the presence of IFN-specific sequences by an assay that depends upon hybridization of IFN mRNA to plasmid DNA (Maeda et al. 1980; McCandliss et al. 1981b). Plasmid DNA from ten recombinants was isolated and covalently bound to diazobenzloxymethyl (DBM) paper. The mRNA from induced cells was hybridized to each filter. Unhybridized mRNA was removed by washing. After the specifically hybridized mRNA was eluted, both fractions were translated in *Xenopus laevis* oocytes. Once a positive group had been found (one in which the specifically hybridized mRNA yielded IFN after microinjection into frog oocytes), it was necessary to identify the specific clone or clones containing IFN cDNA. The individual colonies were grown, the plasmid DNAs were prepared, and each individual DNA was examined by mRNA hybridization as above. By these procedures, a recombinant, plasmid 104 (p104), containing most of the coding sequence for a Hu-IFN-α, was identified (Maeda et al. 1980). The DNA sequence was determined and found to correspond to what was then known of the amino acid sequence of purified Hu-IFN-α (Levy et al. 1980; Levy et al. 1981). The cDNA insert in plasmid p104 contained the sequence corresponding to more than 80% of the amino acids in IFN-αA, but not for those at its amino-terminal end. It was, therefore, used as a probe for finding a full-length copy of the IFN cDNA sequence that could be used for expression of Hu-IFN-αA in *E. coli* (Goeddel et al. 1980b). In addition, p104 DNA was used to isolate DNA sequences corresponding to other IFN-α species directly from a human gene bank.

Examination of the coding regions of the IFN-α genes that have been isolated in our laboratory and others have shown that these correspond to a family of homologous proteins (Pestka 1983a; Rubinstein et al. 1979c) that are closely related to each (Table 2). Thus, the previously discovered heterogeneity in Hu-IFN-α was at least in part the result of distinct genes representing various expressed Hu-IFN-α sequences. The cloned Hu-IFN-αA (Hu-IFN-α2a), the first one we isolated, corresponds to one of the natural Hu-IFN-αs that we purified by HPLC. By procedures similar to those described for plasmid p104, plasmid p101 was shown to contain the sequence for Hu-IFN-β. Thus, the nucleotide sequences coding for Hu-IFN-α and Hu-IFN-β were identified.

## 4
## The Recombinant Human IFN-α Genes and Proteins

A summary of the IFN-α genes and proteins is listed in Table 2. There are in essence 14 human genes that comprise the IFN-α family. Minor variants consisting of one or two amino acid differences account for the multiple alleles (Diaz et al. 1994; Krause and Pestka 2005; Pestka 1983a, 1983b, 1986). Excluding the pseudogene IFNAP22, there are 13 genes. One of them, IFNA10 is also a pseudogene in one allelic form. There are 13 proteins expressed from these genes. The protein produced from gene IFNA13 is identical to that produced from IFNA1. Thus, there are 12 separate IFN-α proteins (and allelic forms) produced from these 14 genes (Table 2).

## 5
## Concluding Summary

This review concentrated on the purification and cloning of IFN-α. Several extensive reviews (Pestka 1983a, 1983b, 2000) provide further details with tables and figures that would be useful to the reader. The recombinant proteins produced in *E. coli* have properties substantially equivalent to the proteins produced by human cells. Predominantly, only one recombinant IFN-α protein is used therapeutically (IFN-α2a, IFN-α2b, and IFN-αc, allelic variants) so that the remaining IFN-α species remain an untapped reservoir of opportunity. Why the body produces so many of these interferons remains unanswered. As our understanding of the mechanism of their receptor interactions develops, some of these answers should be forthcoming.

Although purification of the interferons to homogeneity remained elusive for about two decades after their discovery, they are now available in purified form. The availability of these proteins for laboratory and clinical studies has already catalyzed extensive new developments with these agents and it is likely that we will gain new insights into their actions and develop new applications for their use in the near future.

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