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Interferon-γ binds to heparan sulfate by a cluster of amino acids located in the C-terminal part of the molecule

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Using three different approaches (domain mapping with monoclonal antibodies, limited enzymatic digestion and competition with synthetic peptides), we demonstrated that a cluster of basic amino acids on interferon-γ is involved in its binding to heparan sulfate. This cluster (Lys^{131}–Arg^{141}) is localized in the C-terminal part of IFN-γ. Once bound to heparan sulfate, IFN-γ is protected against protease attack.

Interferon-γ; Heparan sulfate; Heparin; Binding site; Molecular stability

1. INTRODUCTION

Interferon-γ, one of the three major classes of interferon (IFN), is a glycoprotein product of activated T-lymphocytes. This 143 amino acid polypeptide is involved in the regulation of many facets of the immune response, and has antiviral, antiproliferative, and antitumoral activity in a great number of cells [1]. The first step of IFN-γ action is its binding (K_d = 10^{-9}–10^{-11} M) to a specific cellular membrane receptor [2].

Recently, we have shown that IFN-γ also binds to basement membranes with a great affinity (K_d = 10^{-9} M). This interaction could be biologically relevant since basement membranes could provide a local concentration of this soluble cytokine, direct the range of its action and act as a physiological storage depot around cells [3,4]. This basement membrane-binding site was found to be on the heparan sulfate proteoglycan carbohydrate side chains. Once bound to the basement membrane's heparan sulfate (HS), IFN-γ retains full antiviral activity [4]. Furthermore the complex HS/IFN is also active, even in a 1000-fold excess of HS (manuscript in preparation), suggesting that IFN-γ binds to its cellular receptor and to HS by distinct amino acids. The present study was undertaken to determine the amino acids on IFN-γ involved in this binding.

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Abbreviations: IFN, interferon; Mab, monoclonal antibody; HS, heparan sulfate; HP, heparin

2. MATERIALS AND METHODS

2.1. *Domain mapping with monoclonal antibodies (Mabs)*

Small strips of nitrocellulose dot blotted with 1 μg of HS (Sigma) were incubated overnight at 4°C in 500 μl of binding buffer (PBS/BSA 1%), containing 0.25 μg of IFN-γ. After 5 washes in PBS, the nitrocellulose strips were incubated for 1 h at room temperature with either polyclonal anti-IFN-γ antibody 1/200 (Genzyme) or various Mabs to IFN-γ 1/10 (UNICET). A peroxidase-labelled second antibody was used to reveal nitrocellulose strips as described [3]. The Mabs, B22, B27, 32 and 35, recognize different epitopes along the IFN-γ molecule and are described elsewhere [5].

2.2. *Enzymatic digestion of IFN-γ*

IFN-γ was digested with either trypsin, clostripain (as described in [6,7]) or with carboxypeptidase Y at a 1:10 molar ratio for 30 min at 25°C. Each digest was performed with or without HS (IFN/HS molar ratio = 0.4), and analysed by a 15% SDS-PAGE.

2.3. *Synthetic peptides* Peptide AKTGKRKRSG (referred to as P1 in the text) was synthesized with the Biolinx 4175 peptide synthesizer (Pharmacia) using ultrosyn A resin and Fmoc amino acid derivatives. The purity of the peptide was checked by HPLC and amino acid analysis. Peptide RGRR (P2) was obtained commercially (Neosystem).

2.4. *Competition study*

Dot-blotted HS (1 μg) was incubated with 0.1 ng of ^{125}I-radiolabelled IFN-γ (Amersham) and with increasing concentrations

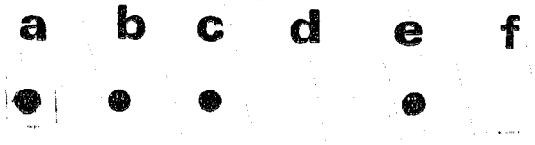

Fig. 1. Domain mapping of IFN-γ. Dot-blotted HS was incubated first with IFN-γ (a–e) or PBS/BSA (f), then with Mabs B27 (a), 32 (b), 35 (c), B22 (d) or polyclonal anti-IFN-γ (e,f), and finally with peroxidase-labelled anti-IgG (a–f).

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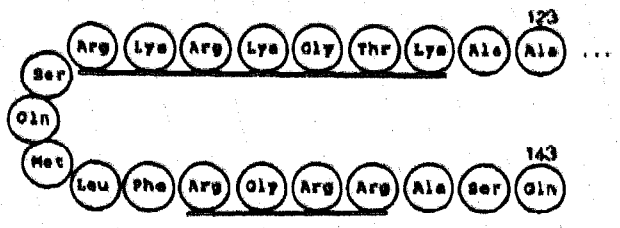

Fig. 2. Amino acid sequence of the C-terminus of IFN-γ (from [1]). The two basic clusters suspected for HS binding are underlined.

of various competitors: IFN-γ, synthetic peptides both alone or in combination. After washing, nitrocellulose strips were directly counted, and the results were calculated as a percentage of remaining bound IFN-γ.

3. RESULTS

3.1. *IFN-γ domain mapping*

We first determined which sites on the IFN-γ/HS complex were not exposed, using monoclonal antibodies to IFN-γ. IFN-γ was first allowed to react with dot-blotted HS and then incubated with different antibodies. As shown in Fig. 1a-c, Mabs B27, 32 and 35 gave a positive signal, showing that these Mabs can bind to HS-bound IFN. In contrast, B22 (the only Mab recognizing the C-terminal part of IFN) gave a negative signal, suggesting that HS and B22 compete for the same binding site on IFN, and thus that IFN-γ binds to HS via its C-terminus. The C-terminus of IFN-γ contains two clusters of basic amino acids (Fig. 2) which both could be involved in the binding.

3.2. *Enzymatique digestion*

Limited digestion of IFN-γ with trypsin or clostripain results in a peptide fragment of $M_r \sim 15\,500$ (Fig. 3, lanes c and d). The cleavage sites of these 2 enzymes are respectively Lys^{130}-Arg^{131} and Arg^{129}-Lys^{130} [6,7], and are thus located in the first basic cluster. Carboxypeptidase Y removed amino acids sequentially from the C-terminus, and under our conditions give a protein of $M_r \sim 16\,000$ (lane e), which is consistent with the removal of at least 10 amino acids. Thus, the second basic cluster (Fig. 2) is likely removed by this treatment. In the presence of HS, the digestion of IFN by these 3 enzymes is prevented (Fig. 3) suggesting that IFN-γ may bind or at least interact with HS by these 2 clusters.

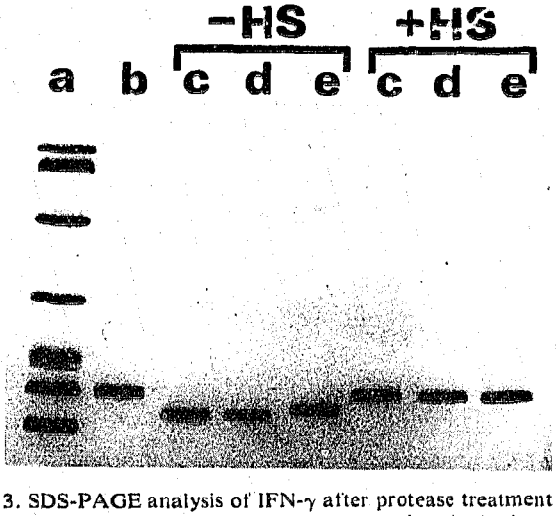

Fig. 3. SDS-PAGE analysis of IFN-γ after protease treatment in the presence or absence of HS by: trypsin (lane c), clostripain (lane d) and carboxypeptidase Y (lane e); molecular weight markers: 97.4, 66.2, 42.7, 31, 21.5, 17, and 14.4 kDa (lane a), intact IFN-γ (lane b).

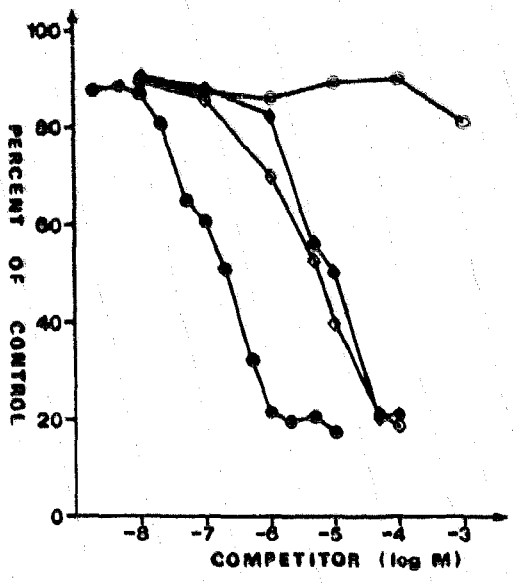

Fig. 4. Competitive binding of IFN-γ and synthetic-peptides against ^{125}I-IFN for HS. IFN-γ (•), AKTGKRKRSG (♦), RGRR (⊜), and equimolar mixture of both peptides (○).

3.3. *Competition study*

To more directly assess the importance of these two domains in the binding, competitive experiments were carried out with two synthetic peptides AKTGKRKRS (P1) and RGRR (P2) corresponding to these two clusters. As shown in Fig. 4, P1 and IFN-γ compete with radiolabelled IFN for the binding to HS, whereas P2 does not. Furthermore a mixture of P1 and P2 does not significantly compete more than P1 alone with ^{125}I-IFN. This demonstrates that the sequence Lys^{125}-Arg^{131} is involved in the binding of IFN-γ on HS, but the sequence Arg^{137}-Arg^{140} probably contributes less to the attachment of interferon to heparan sulfate.

4. DISCUSSION

The data here demonstrate that IFN-γ binds to HS by a cluster of basic amino acids located in the C-terminal part of the protein. The C-terminal part of IFN-γ is surrounded by solvent molecules [6], and is therefore extremely sensitive to protease attack [6,8]. Even in

absence of proteases, the C-terminus is very unstable [8]. IFN-γ lacking its C-terminal 19 amino acids has a higher T_m than the full-length molecule in reversible thermal unfolding [9]. Thus, it has been suggested that the C-terminus peptide undergoes an unfavorable, destabilizing interaction with the rest of the IFN-γ structure. We would like to suggest that the use of HS or HP (or fractions of these) could be a valuable means of stabilizing (by neutralizing the highly positive charge of the C-terminus) and protecting (against protease attack) the IFN-γ molecule, especially in the therapeutic use of this cytokine.

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