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Analysis of murine interferon-γ binding to its receptor on intact cells and solubilized membranes

Identification of an 80 kDa receptor

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The receptor for murine-interferon-γ (Mu-IFN-γ) has been characterized for its molecular size and equilibrium binding constant on a thymoma cell line, EL-4. Binding of ^{125}I-IFN-γ to intact cells and their solubilized membranes has shown a single class of receptor with K_d values of 1.9×10^{-9} M and 1.3×10^{-8} M, respectively. It was shown that solubilization of the Mu-IFN-γ receptor with a Zwitterionic detergent (Chaps) preserves its binding activity. A direct comparison of the molecular mass of the Mu-IFN-γ receptor on intact cells versus detergent-solubilized membranes was performed using a radiolabeled photoactivated crosslinking reagent and direct hybridization with ^{125}I-labeled IFN-γ on Western blots of solubilized receptor. The results indicate that both types of receptors have an identical molecular mass of ~80 kDa.

Interferon-γ receptor; Detergent solubilization; Equilibrium-binding constant

1. INTRODUCTION

IFN-γ, a lymphokine produced by T lymphocytes upon antigen stimulation, is an important modulator of the immune response [1–3]; it also possesses antiviral and anti-proliferative activities [4]. The receptor(s) for IFN-γ display(s) high-affinity binding sites, with dissociation constants ranging from 10^{-9} to 10^{-11} M and are expressed in varying amounts (2000–60000 sites per cell) on all cell lines so far tested [5,6]. The biochemical characterization of human and murine IFN-γ receptors has revealed a single glycoprotein of 90–95 kDa [7–10]. In this report, we identify in the murine thymomas, EL-4, a single receptor of 80 kDa present in both intact cells and detergent-solubilized membranes.

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2. EXPERIMENTAL

2.1. *Membrane preparation and solubilization*

2×10^9 EL-4 cells were washed in PBS and placed in 20 ml of a hypotonic solution (10 mM NaCl, 10 mM Tris, 0.2 mM $CaCl_2$, 1.5 mM $MgCl_2$) plus the following protease inhibitors in μg/ml: 35 PMSF, 10 TLCK, 50 TPCK, 2 aprotinin, 0.7 pepstatin, 0.5 leupeptin for 20 min at 4°C and stirred. Complete cell lysis was then obtained using a nitrogen cavitation bomb at 15 Atm for 30 min. Membranes were recovered with sequential centrifugation of the supernatant at $1000 \times g$ for 7 min, $3500 \times g$ for 10 min and $40000 \times g$ for 1 h. The final pellet was solubilized in 2 ml of PBS, 8 mM Chaps (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate) plus the protease inhibitors mentioned above. Solubilized membranes were clarified at $100000 \times g$ for 1 h and stored at −70°C at ~2 mg/ml.

2.2. *Binding on intact cells*

Triplicates of 1×10^6 EL-4 or in some experiments 2.5×10^6 WEHI-3 cells were incubated in 300 μl of RPMI 1640, 0.2% BSA for 16 h at 4°C by shaking with 50 pM of recombinant ^{125}I-IFN-γ and with different amounts of cold ligand (100 pM to 500 nM). IFN-γ was a gift of Drs Nobuo Yoshida and Jenji Sugita, Shionogi Research Laboratories, Osaka, Japan. Separation between free and bound ^{125}I-IFN-γ was accomplished as

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described [11]. Binding data were analyzed with the LIGAND program [12].

2.3. *Binding to solubilized membranes*

20 μl of solubilized membranes were incubated at room temperature in 220 μl of PBS with the addition of 500 pM ^{125}I-IFN-γ with or without cold ligand at concentrations between 300 pM and 1 μM. After 1.5 h, the separation between free and bound ^{125}I-IFN-γ was accomplished as described [13].

2.4. *Photocrosslinking of IFN-γ with HSAB*

The photocrosslinking protocol was modified from Hosang and Shooter [14]. Briefly, 10^7 EL-4 cells were incubated in 1 ml for 16 h at 4°C by shaking with 10 pmol of ^{125}I-IFN-γ. Cells were washed thrice in RPMI and then incubated in 1 ml of PBS with 0.5–500 μM HSAD (*N*-hydroxysuccinimidyl-4-azidobenzene, Pierce Chem. Co.) for 1 h on ice in the dark. Cells were irradiated at 366 nm for 10 min, solubilized and the supernatant run on SDS-PAGE.

2.5. *Photocrosslinking of IFN-γ with Denny-Jaffe reagent*

0.5 mCi of ^{125}I-labeled Denny-Jaffe reagent (*N*-[4-*p*-azido-*m*-^{125}I]iodophenylazo)benzoyl]-3-aminopropyl-*N'*-oxysuccinimide ester, New England Nuclear, SA, 2200 Ci/mmol) and 10 μg of IFN-γ were conjugated in borate buffer, pH 8.5, for 2 h on ice. 300000 cpm of the conjugated IFN (SA 2 μCi/μg) was incubated with 10^7 EL-4 cells for 3 h with or without 100-fold excess of cold ligand. After washing in PBS, cell pellets were irradiated at 365 nm for 8 min. Cells were centrifuged at 500 × *g* for 1.5 min and resuspended in 100 μl of 0.2 M sodium dithionite, pH ~7.5, for 20 min on ice, then centrifuged. The pellet and supernatant were analyzed by SDS-PAGE.

2.6. *Gel electrophoresis and Western blot*

10 μl (2 μg/μl) of solubilized membranes were mixed with an equal volume of 2.5 mM Tris-Cl, pH 6.8, containing 8 mM Chaps and 20% glycerol and loaded without reduction and boiling onto a 7.5% polyacrylamide gel to prevent denaturation of the receptor. The stacking and separating gels contained Chaps instead of SDS; the electrode buffer contained 0.1% SDS. Following Western blotting, the nitrocellulose blots were saturated for 2 h in PBS, pH 8.3, and 0.05% Tween 20 (buffer A) with 10% nonfat dry milk. ^{125}I-IFN-γ (~300000 cpm in 1 ml) in buffer A and 1% nonfat dry milk, was incubated for 2 h with or without 50-fold excess of cold IFN-γ. After 4 washes with buffer A containing 10% nonfat dry milk, the dried blot was subjected to autoradiography.

3. RESULTS AND DISCUSSION

3.1. *Binding of IFN-γ to EL-4 cells*

The thymoma cell line, EL-4, was chosen for these studies because it displayed a relatively high number of receptors per cell [5,6,15]. Equilibrium binding analysis of the IFN-γ receptor on these cells gave a dissociation constant of 1.9×10^{-9} M ± 10% as determined by competition studies with unlabeled ligand (fig.1A). WEHI-3 cells were also examined and exhibited a K_d of 6×10^{-9} M with 13000 receptors per cell (not shown). WEHI-3 and other cells of macrophage origin are reported to express two different affinity binding sites for Mu-IFN-γ [11], whereas the linear characteristics of our binding data suggests the existence of a single class of noncooperative binding sites on lymphoid and on macrophage cell lines.

3.2. *Binding to solubilized membranes*

The Chaps-solubilized EL-4 receptors had a K_d of 1.3×10^{-8} M and the amount of receptor was 26.5 pM/mg of total solubilized protein (fig.1B). The lower affinity of the solubilized receptor most likely is the result of a partial loss of binding affinity upon solubilization, as described for Mu-IFN-γ solubilized by octylglucoside [9] and other receptors [16]. The EL-4 receptor apparently is sensitive to the hydrophobic environment, as is suggested by the loss of about 50% activity if membranes are solubilized with Triton X-100 rather than Chaps (not shown).

3.3. *Crosslinking with photoactivable reagents on intact cells*

To investigate the molecular size of the Mu-IFN-γ receptor as well as the possible existence of more than two different receptor species, crosslinking experiments were performed. Photoactivated reagents have been shown to resolve receptor molecules which have different affinity for the same ligand [14]. Cells were completely saturated with IFN-γ for 16 h and then incubated with different concentrations of the photoactivated crosslinking reagent, HSAD. Under reducing conditions at concentrations from 0.5 to 50 μM, only a single band of about 95 kDa was detected, whereas at 500 μM HSAD two bands of 95 and 110 kDa were observed (fig.2A). Since saturating levels of ligand were used under equilibrium binding conditions, if two receptors exist, then both of these bands should be present at the lower concentrations of crosslinking reagent. Thus, these results suggest that labeled IFN-γ by itself can bind a single receptor molecule either as a monomer (16 kDa) or as a dimer (32 kDa) internally crosslinked by high concentration of HSAD. Alternatively, this higher molecular mass band could represent receptor of identical affinity but which is glycosylated to a greater degree.

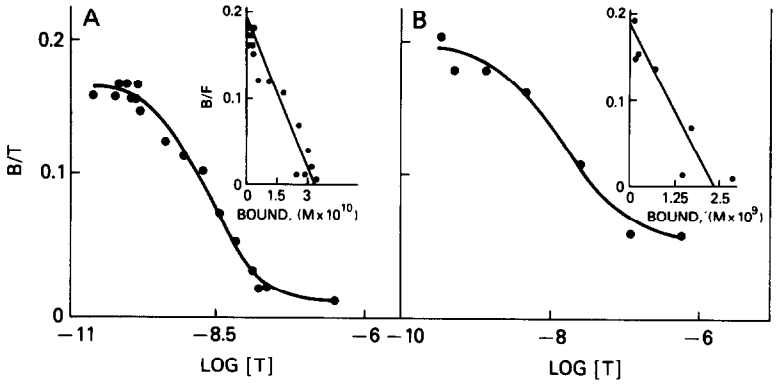

Fig.1. Equilibrium binding analysis of IFN-γ to EL-4 and detergent-solubilized membranes. Binding analysis was performed on intact cells (A) or solubilized membranes (B) as described in section 2 under equilibrium binding conditions (not shown). Non-specific binding was 1 and 5% in A and B, respectively.

The use of the ^{125}I-labeled Denny-Jaffe reagent conjugated to IFN-γ has the advantage of directly labeling the receptor upon UV irradiation and after reductive cleavage of IFN-γ from the complex, allows the determination of the net molecular mass of the ^{125}I-labeled receptor.

As shown in fig.2B (lanes 2,5), under non-reducing conditions, a broad band from 95 to 115 kDa appeared, corresponding to the complex of IFN-γ and receptor. After partial reduction with sodium dithionite, a new band of ~80 kDa was found (lanes 1,4). This crosslinking could be completely removed by excess cold ligand (lanes 3 and 6).

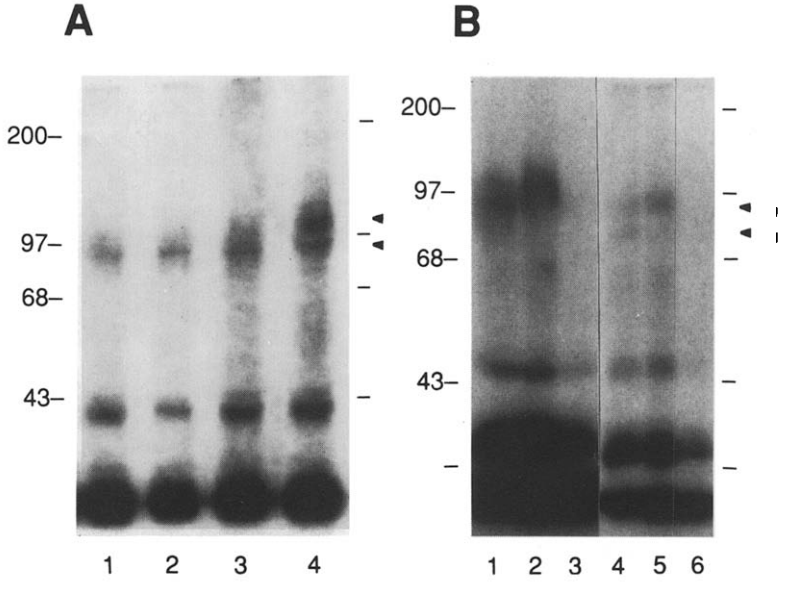

Fig.2. Photochemical crosslinking of IFN-γ to its receptor. (A) ^{125}I-labeled IFN-γ was crosslinked to its receptor on EL-4 cells using HSAB at various concentrations: 0.5, 5, 50 or 500 μM. Results are shown in lanes 1–4, respectively. (B) Crosslinking using the Denny-Jaffe reagent was performed in the absence (lanes 1,2; 4,5) or presence (lanes 3,6) of excess cold IFN-γ as described in section 2. After crosslinking, cells were detergent solubilized and the supernatant (lanes 1–3) and insoluble pellet (lanes 4–6) were analyzed by SDS-PAGE. Lanes 1 and 4 represent partial cleavage of crosslinked IFN-γ receptor complex after dithionite treatment.

3.4. *Western blot and ^{125}I-IFN-γ binding of solubilized receptor*

To compare the biochemical characteristics of solubilized receptors with the molecule expressed on the intact cells, Chaps-solubilized membranes were separated by SDS-PAGE. After blotting of the proteins, ^{125}I-IFN-γ was specifically bound by a protein of about 80 kDa (fig.3). No other bands were detected in this crude preparation of solubilized membranes. This suggests that the only receptor solubilized with Chaps corresponds to the one expressed on the intact cells.

In conclusion, these data demonstrate that the Mu-IFN-γ receptor is a single molecule of ~80 kDa in the EL-4 cell line. The equilibrium binding analysis indicated a single binding site for both EL-4 and WEHI-3 which is consistent with the majority of other reports on mouse and human IFN-γ receptors.

The existence of only one receptor and binding site, however, does not preclude a post-translational modification of the molecule as being responsible for the observed molecular heterogeneity reported in different tissues [17]. In addition, Hayes et al. [9] partially purified and characterized a 95 kDa receptor from WEHI-3 cells, which is 15 kDa larger than the molecule characterized here from the EL-4 cell line. This difference is probably due to varying degrees of glycosylation, however only the cloning of this gene(s) will allow full assessment of any differences which may exist among the receptors on different cell types of the same species.

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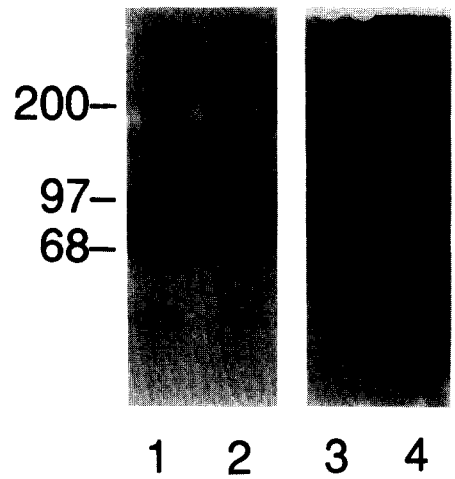

Fig.3. Detection of IFN-γ receptor on Western blots. Receptor preparations were electrophoresed, subjected to Western blotting and blots incubated with ^{125}I-labeled IFN-γ as described in section 2. Blots were incubated in the absence (lanes 1,2) or presence (lanes 3,4) of cold IFN-γ. Lanes 1,3 and 2,4 represent analyses of 40 and 20 μg of membrane protein, respectively.

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