Interferon receptor recognition peptides enhance the biological potency of interferon alphas

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Abstract Based on our earlier studies that defined three strategic regions in the Type 1 interferon (IFN) molecule associated with receptor interactions and biological activity, three IFN receptor recognition peptides (IRRP) were synthesized, with amino acid sequences CLKDRHD (IRRP1), ESLLEKFYTELYQQLND (IRRP2) and YFQRITLYLTEKKYSPCA (IRRP3) and examined for biological effectiveness. In cell surface receptor binding studies, the binding capacity of cells for IFN-αs was increased in the presence of the IRRPs. Increased receptor occupancy resulted in increased phosphorylation-activation of the transcription factor ISGF3 and enhanced antiviral activity. The potentiating effect on IFN-induced growth inhibition was less marked. These data suggest that the IRRPs may influence the biological potency of IFN-α by facilitating accessibility to cell surface receptor components. The IRRPs may function to increase the number of low affinity receptor–ligand interactions necessary to initiate receptor oligomerization, thereby catalysing the formation of high affinity IFN–receptor complexes.

Key words: Interferon-α; Receptor

1. Introduction

Type 1 interferons, IFN-alphas (IFN-α) and -beta (IFN-β), mediate their biological effects on target cells via a high affinity interaction with specific cell surface receptors. Several laboratories have provided evidence that the Type 1 IFN receptor is multimeric, comprising both peptide [1–3] and glycosphingolipid [4] components. To date, the sequence of events that characterize the high affinity interaction between the ligand, IFN, and the different receptor components remain unclear. Undoubtly, the relative abundance of different receptor components will determine the sensitivity of cell types for IFNs. Moreover, there is some evidence to suggest that cells may be variably responsive to IFN-αs vs. IFN-β, dependent on constituent components associated with the receptor complex [5]. Although the existence of an IFN-β-specific receptor component, or alternatively, a receptor configuration that favours a high affinity interaction with IFN-β, have not been shown, studies from this laboratory indicate that a strategic domain within the Type 1 IFN molecule, that is different for IFN-αs and IFN-β, may accommodate such differences in receptor interactions [6,7].

Recent modelling data [7,8] would suggest that three epitopes on the IFN molecule, that have been implicated in influencing receptor interactions and biological activity, are accessible on the surface of the molecule and may, indeed, function as receptor recognition epitopes. The present studies were undertaken to examine the influence of three peptides, that correspond to these strategic domains, on the biological effects of IFN-α. The synthetic peptides used were CLKDRHD (IRRP1), ESLLEKFYTELYQQLND (IRRP2) and YFQRITLYLTEKKYSPCA (IRRP3), that correspond to the putative ligand binding domains assigned to amino acid residues 29–35, 78–95 and 123–140, respectively, of IFN-Con_1. Accumulating evidence suggests that IFN-α/β bind to the IFNABR chain [3] of the IFNABR/IFNAR1 receptor complex, thereby invoking ligand-induced phosphorylation of IFNAR1 [8] and phosphorylation-activation of associated Jak/tyk family kinases [9–11]. The phosphorylated-activated multimeric transmembrane complex is able to sequester and phosphorylate on tyrosine residues a limited panel of related transcription factors, or STAT proteins, among them cytoplasmic Stat1 (p91) and Stat2 (p113) proteins [12]. Stat1 and Stat2 dimerize via SH2 domains, then translocate to the nucleus and associate with an adaptor protein, p48, to form the stable and active transcription complex, ISGF3, that binds to a specific IFN-stimulated response element, ISRE, within the promoter region of IFN-inducible genes. Thus, ligand-induced receptor activation leads to the transcriptional induction of a distinct set of genes that define the characteristic biological responses effected by IFNs [13–21]. We have shown that the extent of receptor occupancy directly correlates with the degree of ISGF3 activation, at least for IFN-αs [4]. Since ISGF3 activation effects the transcriptional induction of IFN-sensitive genes, there is a direct correlation between ISGF3 activation and biological outcome.

In this report we provide evidence that selected IRRPs influence the binding characteristics of IFN-αs such that receptor occupancy increases, leading to enhanced ISGF3 activation and increased biological activity.

2. Materials and methods

2.1. Cell cultures

Human Burkitt lymphoma Daudi cells, were grown as a suspension culture and subcultured in RPMI 1640 medium supplemented with 10% FCS. T98G, human glial and MRC-5, human embryonal lung cells were grown as monolayers in MEM containing 10% FCS.

2.2. Interferons, IRRPs and related reagents

IFN-Con_1, as a consensus IFN-α, was a gift from Amgen Inc. (Thousand Oaks, CA) and had a specific activity of 3.0×10^9 U/mg protein. This synthetic variant was designed to represent an average IFN-α, with an amino acid chosen at each position along the polypeptide that was most frequently represented in the known family of IFN-αs. IFN-α2 was a gift from Schering Canada Inc. and had a specific activity of 2×10^8 U/mg protein. Three IRRPs (US & International PCT Application Serial no. PCT/CA93/00279), with amino acid sequences from N- to C-terminus of CLKDRHD (IRRP1), ESLLEKFYTELYQQLND (IRRP2) and YFQRITLYLTEKKYSPCA (IRRP3) were synthesized, using an Applied Biosystems Model 430A Peptide Synthesizer. Peptides

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Abbreviations: IFN-α; IRRP; STAT; ISGF3; IFNAR1; IFNABR

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were HPLC purified and amino acid composition determined by both amino acid analysis and mass spectrometry. A random hexapeptide, YVKRVK, was a gift from K. Kain (Department of Microbiology, University of Toronto).

2.3. In vitro assays for antiviral and growth inhibitory activities

The in vitro assays for antiviral and antiproliferative activities have been previously described [6].

2.4. Iondination of IFN-α and cell surface receptor binding assays

Labelling of IFN-α2 or IFN-Con_1, with ^{125}I was carried out using the chloramine T method [22]. Iondination caused no detectable loss of activity. The receptor binding assay for suspension cultures has been previously described [23]. With increasing concentrations of [^{125}I]-ligand in the cellular binding reactions, respective specific binding activities corresponding to each [^{125}I]-ligand concentration were calculated.

2.5. Covalent crosslinking of [^{125}I]-IFN-Con_1 to cell surface receptors

Following binding of [^{125}I]-IFN-Con_1 to cells at 4°C for 2 h, bound IFN was crosslinked to the receptors by reaction with 1 mM disuccinimidyl suberate. Cells were washed with PBS and the receptor–ligand complexes extracted with 1% Triton X-100. Soluble extracts were analyzed by SDS-PAGE containing 7.5% acrylamide. Gels were dried and subjected to autoradiography.

2.6. Oligonucleotides

A double-stranded oligonucleotide, representing nucleotides −107 to −87 of the human 2-5A synthetase gene which contains a functional ISRE, was synthesized. The sequence is CCTTCTGAGGAAAGGAAACCA. This oligonucleotide was synthesized with a *Sal*I-compatible linker at the 5′ terminus (TCGAC). Gel-purified oligonucleotide was mixed with an equimolar amount of its respective complement, heated to 65°C for 15 min, and annealed at room temperature for 18 h. This preparation was end-labeled for use in electrophoretic mobility gel shift assays by T4 polynucleotide kinase in the presence of [γ-^{32}P]-ATP.

2.7. Extract preparation and electrophoretic mibility shift assay (EMSA)

Whole cell extracts were prepared from IFN-treated or untreated cells as described in [24,25]. Binding reactions for the gel retardation assay contained 5′ ^{32}P-labeled oligonucleotide, specific competitor DNA, and whole cell extract as indicated in the figure legends. Incubations were at room temperature for 30 min. EMSAs were performed as previously described, using native 6% polyacrylamide gels run in a Tris/glycine/EDTA buffer, pH 8.3 [4].

3. Results

3.1. Receptor recognition properties of IRRP1, IRRP2 and IRRP3

At the outset, studies were conducted to examine the receptor recognition characteristics of the IRRPs. We examined the influence of each of the IRRPs on [^{125}I]-IFN-α2 binding to Daudi cells at 4°C. The results are shown in Fig. 1. Cells were incubated with the indicated doses of [^{125}I]-IFN-α2 in the presence or absence of varying doses of the IRRPs. The experimental conditions were such that the binding reaction reached a steady state equilibrium with respect to bound [^{125}I]-IFN. The amount of bound [^{125}I]-IFN at each input dose of [^{125}I]-IFN is designated 100%. The results demonstrate that each of the IRRPs will affect the binding capacity of the cells for the [^{125}I]-IFN by increasing the total amount of bound [^{125}I]-IFN. It is noteworthy that the IRRPs either alone, or in combination, do not compete with IFN-α2 for binding to the Daudi cells at 4°C. Similar results were obtained when the IRRPs were incubated together with [^{125}]-IFN-Con_1 (data not shown). When [^{125}I]-IFN-Con_1 was cross-linked to cell surface receptors on Daudi cells, either in the presence or absence of each of the IRRPs,

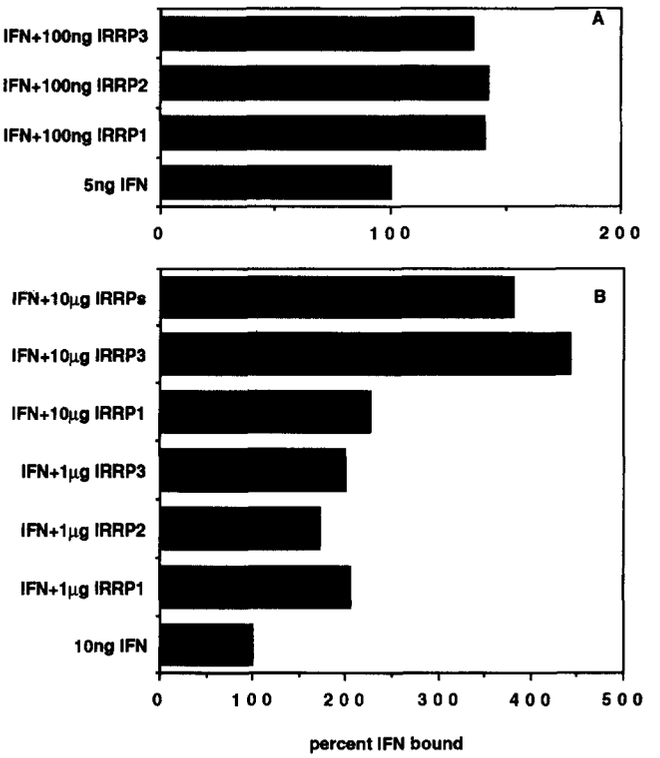

Fig. 1. IRRPs increase the binding capacity of Daudi cells for IFN-α. 5×10^5 Daudi cells were incubated for 2 h at +4°C with either 5 ng (A) or 10 ng (B) of [^{125}I]-IFN-α2, with or without the indicated concentrations of IRRPs. The values shown were obtained by subtracting non-specific counts/min bound from total counts/min bound. Non-specific binding was determinded in the presence of a 100-fold excess of unlabeled IFN. Each value represents the mean of triplicate cultures and exhibited a S.E.M. ± 11%.

then the cross-linked complexes separated by SDS-PAGE, similar banding patterns were observed (Fig. 2).

3.2. Influence of IRRPs on IFN-induced ISGF3 activation

Since transcription factor activation is an early event that immediately follows the interaction between IFN and the receptor complex, experiments were undertaken to examine the effect of IRRP treatment on IFN-induced ISGF3 activation in different cells. Cells were treated with IFN-Con_1, an IRRP, or a combination of IFN-Con_1 and IRRP. Cell extracts were prepared and ISGF3 activation determined in the context of a shift in mobility of an ISRE in an electrophoretic gel shift assay. The results in Fig. 3A demonstrate that IRRP1, IRRP2 and IRRP3 treatment of Daudi cells did not lead to ISGF3 activation. By contrast, IFN-Con_1 treatment of the same cells resulted in ISGF3 activation (Fig. 3A). However, when Daudi cells are treated with IFN-Con_1 together with either IRRP1 or IRRP2, the extent of ISGF3 activation is increased compared with the level of ISGF3 activation observed with IFN-Con_1 alone (Fig. 3B). These results are consistent with the observation that these IRRPs increased receptor occupancy by IFN on these cells, i.e. receptor occupancy determines the extent of transcription factor activation.

MRC-5 cells exhibit a limited sensitivity to the antiviral effects of IFNs-α/β. Sensitivity to the effects of IFN correlates

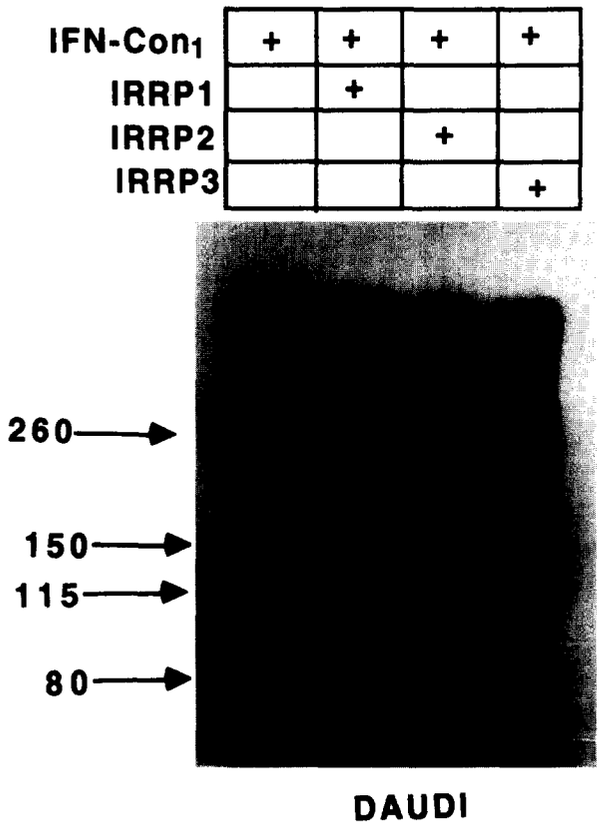

Fig. 2. IRRPs do not affect the affinity characteristics of [^{125}I]-IFN-Con$_1$ on Daudi cells. Covalent crosslinking of [^{125}I]-IFN-Con$_1$ to Daudi cell surface receptors. Cells were incubated with 25 ng/ml of [^{125}I]-IFNCon$_1$ in the presence or absence of 1 μg/ml each of IRRP1, IRRP2 and IRRP3, at +4°C for 2 h, then bound IFN was crosslinked to the receptors and analyzed by SDS-PAGE and autoradiography. Positions of ^{14}C-labeled molecualr weight markers, expressed in kDa, are shown.

with the limited binding capacity of these cells for IFNs-α/β, despite evidence for good cell surface expression of the two receptor chains IFNAR1 and IFNABR, and the limited activation of ISGF3 (unpublished observations, manuscript submitted). Since the preceding data would suggest that the IRRPs enhance IFN-inducible activation of ISGF3, we examined whether IRRP1 would have the same effect on MRC-5 cells. The results in Fig. 3C demonstrate that IRRP1 treatment of MRC-5 cells enhances IFN-Con$_1$-inducible ISGF3 activation.

3.3. *Influence of IRRPs on IFN-induced antiviral and growth inhibitory activities*

In the final series of experiments we examined the effects of IRRP treatment on IFN-induced biological activities. Fig. 4A describes the results obtained when human glial T98G cells are challenged with EMCV, either in the absence or presence of IFN-Con$_1$ and the IRRPs. The protective effects of sub-optimal doses of IFN-Con$_1$ on viral CPE are increased in the presence of IRRP1, IRRP2 and IRRP3. Each of the IRRPs confers no protection from viral infection when used alone. These results are in agreement with both the binding and signaling data: IRRP-mediated increases in IFN receptor occupancy leads to increased IFN-induced transcriptional activation and increased antiviral potency of the IFN. Earlier studies from this laboratory have demonstrated that the extent of IFN-induced ISGF3 activation correlates well with antiviral responses, yet is not linked to IFN-induced growth inhibition (manuscript submitted). Moreover, there is general agreement that the threshold dose for an IFN-induced growth inhibitory response is significantly higher than that for an antiviral response. Accordingly, we examined the influence of IRRP1 treatment on IFN-induced antiproliferative responses in the T98Gs, employing a 10-fold higher dose of IRRP1 than was used in the antiviral studies. The results in Fig. 4B show that IRRP1 marginally increase the growth inhibitory activity of IFN-Con$_1$, under the prescribed conditions. The most pronounced effects were obtained when the lower doses of IFN were used.

4. Discussion

IFN-induced biological responses require the interaction between IFN molecule(s) and the large, transmembrane multimeric receptor complex. Although sites of interaction between ligand and receptor have not been assigned, an emerging consensus is that different faces of the IFN molecule interact with independent sites on the reeptors. Factors that contribute toward the affinity of the ligand for the multicomponent receptor system include the configuration of the receptor system and the association of specific janus kinases with the intracellular regions of the receptors. The overall avidity of complex formation depends on the crowding of receptors at the cell surfac and the flux of IFN molecules towards these receptors. Our working hypothesis has been that distinct receptor recognition domains on the IFN molecule interact with distinct contact sites on the receptor components. Low affinity interactions between individual recognition epitopes on the IFN molecule and single receptor sites are non-productive in terms of signal transduction. The productive, high affinity binding paradigm we invoke would require simultaneous contacts between the different epitopes on the IFN molecule and their associated receptor components. Thus, both receptor and ligand concentration contribute toward the kinetics of interactions and determine the affinity of complex formation. To test this hypothesis we have synthesized a panel of peptides that correspond to putative receptor recognition domains in the IFN molecule. For a finite number of IFN receptors expressed at the surface of a given cell population, it should be possible to increase the binding capacity of these cells for whole IFN molecules by increasing the flux of contributing receptor binding peptides, thereby increasing the likelihood of successful simultaneous 'hits' on the receptors. Since the intact, native IFN molecule should be optimally configured to bind in the corresponding receptor pockets with high affinity characteristics, it is unlikely that individual peptides will compete for IFN–receptor site binding.

In direct binding studies we measure a partition of the [^{125}I]-IFN between the unbound/free state and the complexed/bound state on the cells. Our experimental conditions are set to measure this partition when the binding reaction has reached equilibrium, i.e. steady state. For different [^{125}I]-IFN-α input concentrations we observed that the IRRPs increased the amount of bound [^{125}I]-IFN to cell surface receptors. Since the binding reactions are conducted at 4°C, the ligand–receptor complexes are not internalized, thus the number of receptors expressed at the surface of the cells is not reduced during the course of the binding experiment. We infer, therefore, that the increase in binding capacity that we observed in the presence of the IRRPs

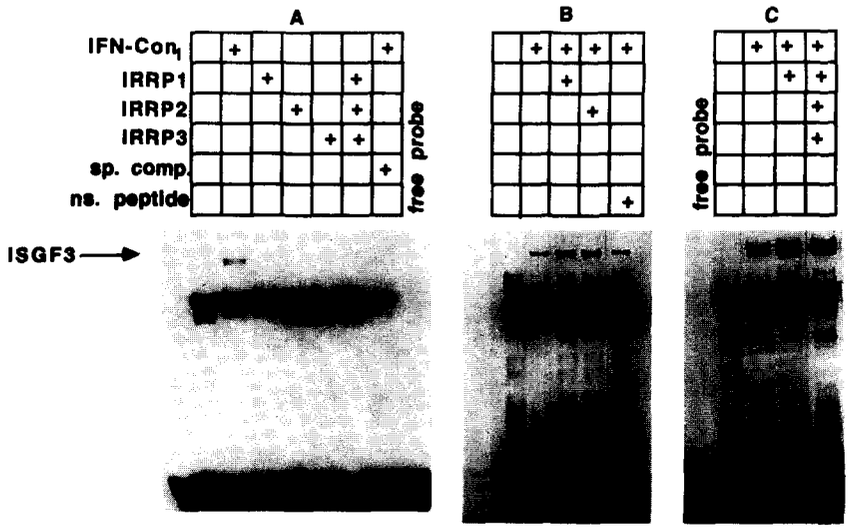

Fig. 3. Influence of IRRPs on IFN-induced ISGF3 activation. Daudi (A, B) and MRC-5 (C) cells were either left untreated or treated with 0.1 ng/ml (A), 0.016 ng/ml (B) or 1.0 ng/ml (C) IFN-Con_1, in the presence or absence of 1 μg/ml of the IRRPs as indicated. 5 μg of whole cell extracts were reacted with 30,000 cpm of a ^{32}P end-labeled ISRE, representing nucleotides −107 to −87 of the human 2-5A synthetase gene, which contains a functional ISRE. Complexes were resolved by using native gel electrophoresis and visualized by autoradiography. Mobility of ISGF3 is indicated. Specific complexes were identified by the addition of a 100-fold excess of unlabeled ISRE (sp. comp.). A random hexapeptide (YVKRVK) was also included. The mobility of the free probe is shown.

is a direct consequence of their influence on the avidity of the interaction between the IFN molecules and the cell surface receptors. The contributing effects of the IRRPs on IFN binding most likely include reducing the dissociation of the ligand, and enhancing IFN–receptor affinity and receptor–receptor interactions. Since, in cross-linking experiments we have demonstrated that the IRRPs do not affect the nature of the multicomponent IFN–receptor complexes that form, these data suggest that the IRRPs may function to catalyse the formation of high affinity IFN–receptor complexes by increasing the number of low affinity receptor–ligand interactions necessary to initiate the cooperativity between receptor components.

The results in Fig. 2 demonstrate that the increase in receptor occupancy mediated by treatment with the different IRRPs results in enhanced activation of the cytoplasmic signaling factor ISGF3. Since IRRP treatment alone was insufficient to activate signal transduction, these data suggest that ligand-induced association of receptor components, that is a prerequisite for activation of receptor-associated tyrosine kinases and/or STAT proteins, involves the modification of multiple receptor sites. That is, the individual receptor components may interact with distinct domains of the IFN molecule, or IRRPs that correspond to these recognition epitopes, but functional activation of the receptor requires that multiple receptor sites are ligand-bound to permit receptor components to complex. Apparently, even a combination of all 3 IRRPs will not facilitate receptor complexing.

MRC-5 cells exhibit a poor binding capacity for IFNs-α/β, that dictates the relatively weak sensitivity of these cells to the biological effects of IFNs. Based on our experimental results with the IRRPs, we investigated whether it would be possible to increase the sensitivity of MRC-5 cells to the effects of IFN by co-treatment with an IRRP. We provide evidence that administration of IRRP1 together with IFN-Con_1 substantially increased the level of ISGF3 activation in MRC-5 cells above that attained with IFN alone. Moreover, we have shown that the effects of these IRRPs on IFN-induced responses are specific, since a random hexapeptide did not affect the level of IFN-induced ISGF3 activation in Daudi cells (Fig. 2B), nor did BSA (data not shown).

Consistent with their ability to potentiate receptor binding and ISGF3 activation effected by IFN, the IRRPs will increase the magnitude of the antiviral response induced by IFN. IRRP1 potentiation of the IFN-induced antiproliferative response was less marked. These data are consistent with observations that the extent of IFN-induced ISGF3 activation directly correlates with the magnitude of the IFN-induced antiviral response, yet does not equate with the extent of IFN-induced growth inhibition. Thus, modification of the IFN–receptor interaction by IRRP treatment, that results in increased receptor occupancy, elicits an early post-receptor influence on signal transduction that ultimately results in an increased biological response. Obviously, the challenge is to design specific IRRPs that maximally affect strategic biological responses, dependent on their modulation of specific receptor components. Preliminary indications are that IFNAR1 activation is tightly linked to signaling events that mediate an antiviral response and that IFNABR is the primary receptor binding component of the receptor complex. Studies are in progress to elaborate the specific amino acid clusters in the IFN molecule that interact directly with the extracellular regions of the different receptor chains. Utilising different IRRPs in combination with IFN we are examining the biological outcomes on cells that variably express the different Type 1 IFN receptor components at their surface. This approach will identify critical receptor recognition domains in the IFN molecule that interact with distinct receptor components.

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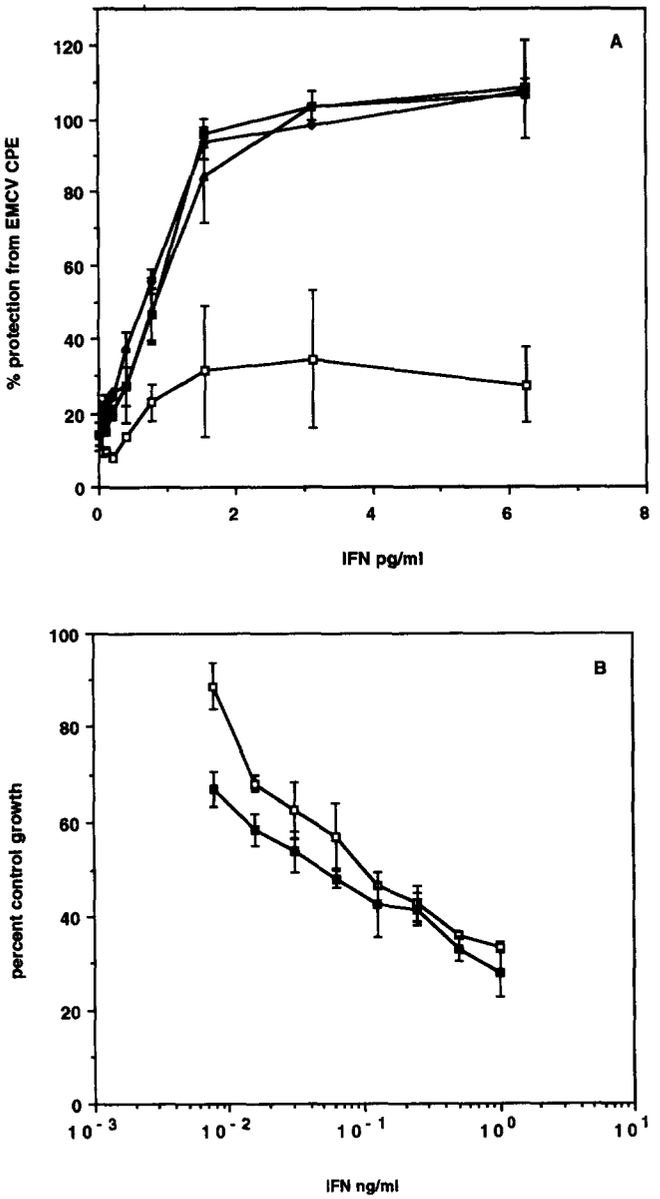

Fig. 4. Influence of IRRPs on IFN-induced biological responses. (A) IFN-induced antiviral activity. T98G cells were left untreated, or treated with IFN-Con_1 in the presence or absence of the different IRRPs. The cultures were then challenged with EMCV and viral CPE quantitated 24 h later. Values are the mean of triplicate determinations and error bars denote the S.D. from the mean. □, IFN-Con_1; ■, IFN-Con_1 + 1 μg/ml IRRP1; ◇, IFN-Con_1 + 1 μg/ml IRRP2; △, IFN-Con_1 + 1 μg/ml IRRP3. (B) IFN-induced antiproliferative activity. T98G cells were treated with IFN-Con_1 (□) or IFN-Con_1 + 1 μg/ml IRRP1 (■) for 96 h at 37°C. IFN-induced growth inhibition was recorded relative to the growth of untreated cultures. Values represent the mean of triplicate cultures and error bars denote the S.D. from the mean.

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