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Induction by interleukin-6 of interferon regulatory factor 1 (IRF-1) gene expression through the palindromic interferon response element pIRE and cell type-dependent control of IRF-1 binding to DNA

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The effects of interleukin-6 (IL-6) on interferon regulatory factor 1 (IRF-1) gene expression were studied in B-hybridoma B9 cells which are growth-stimulated by IL-6 and breast carcinoma T47D cells which are growth-inhibited. IL-6 induced the production of IRF-1 mRNA and protein in both cell types, but IRF-1 binding activity to its target DNA sequence was induced only in T47D cells. With B9 cells, there was no IRF-1 binding but instead strong constitutive binding of the IRF-2 repressor, indicating that binding of IRF-1 to DNA is an important regulatory step. The IRF-1 gene promoter element, palindromic IFN-response element (pIRE), was found to respond to IL-6 with high efficiency as compared with IFN-γ or IFN-β. On this palindromic TTC...GAA sequence, two protein complexes (pIRE-a and pIRE-b) were induced within minutes by IL-6. pIRE-b is similar to the main complex induced by IFN-γ and contains the Stat91 protein. pIRE-a predominantly induced by IL-6 is a slowly migrating complex which does not contain Stat91 and has low affinity for IFN-γ activated sequence (GAS)-type sequences. Comparison of the relative effects of IL-6 and IFN-γ shows that pIRE enhancers are differently regulated than GAS elements. Distinct transcription complexes, forming in ratios dependent on the inducer, help explain how various cytokines sharing effects through Stat91 on related enhancers can produce specific patterns of gene expression. Activation of the pIRE-a factors defines a novel transcriptional activity of IL-6 in epithelial and lymphoid cells.

Key words: interferon regulatory factor/interleukin-6/pIRE

Introduction

Interferon regulatory factor 1 (IRF-1) is a transcription factor regulating the interferon-β (IFN-β) gene and also the action of IFNs on cells via the induction of IFN-responsive genes (Miyamoto *et al.*, 1988; Harada *et al.*, 1990; for reviews see Stark and Kerr, 1992; Tanaka and Taniguchi, 1992). IRF-1 binds to $(GAAAGT)_n$ motifs found in the IFN-β gene promoter (Fujita *et al.*, 1987) and to GAAACC/T repeats (MacDonald *et al.*, 1990) such as found in IFN-stimulated response enhancers (ISRE). Reporter genes containing IRF-1-binding sites are activated by virus infection and by transfection with IRF-1 cDNA vectors (Harada *et al.*, 1990; MacDonald *et al.*, 1990). The function of IRF-1 is inhibited by the related IRF-2 protein which has a similar DNA-binding site but acts as a transcriptional repressor (Harada *et al.*, 1989, 1990; Tanaka *et al.*, 1993). Viruses, double-stranded RNA and IFNs regulate IRF-1 synthesis (Miyamoto *et al.*, 1988; Harada *et al.*, 1989; Pine *et al.*, 1990) but an additional post-translational activation of IRF-1 appears to be effected by viruses and double-stranded RNA (Watanabe *et al.*, 1991).

Transfections with sense and antisense IRF-1 cDNA have indicated a function of IRF-1 for full induction by IFNs of IFN-responsive genes [including (2′-5′) A synthetase and MHC-I genes] and of the antiviral state (Harada *et al.*, 1990; Chang *et al.*, 1992; Pine, 1992; Reis *et al.*, 1992). Growth inhibition, another common effect of IFNs, can be effected by IRF-1 (Kirchoff *et al.*, 1993). Moreover, expression of IRF-2 in NIH 3T3 cells had oncogenic-type effects on cell growth which were inhibited by IRF-1 expression (Harada *et al.*, 1993).

Increased IRF-1 has been observed preceding growth arrest and terminal differentiation in M1 myeloleukemic cells treated by IL-6, the effect being seen at the IRF-1 mRNA (Abdollahi *et al.*, 1991) and DNA-binding levels (Harroch *et al.*, 1993). M1 cell mutants exhibiting neither growth arrest nor induction of (2′-5′) A synthetase by IL-6 (Cohen *et al.*, 1991), lacked the effect of IL-6 on IRF-1 and had constitutive IRF-2-binding activity (Harroch *et al.*, 1993). Since IRF-1 activation in M1 cells could be part of the differentiation program triggered by IL-6, we examined here whether IL-6 is able to induce IRF-1 in other cell types. Cell lines responding to IL-6 by opposing growth effects were chosen: (i) the human breast carcinoma T47D in which IL-6 reduces growth (Chen *et al.*, 1988; Tamm *et al.*, 1989; Novick *et al.*, 1992) and which are rich in IL-6 receptors (Chen *et al.*, 1991), (ii) the murine hybridoma B9 cells which are dependent on IL-6 for their growth and exemplify the hybridoma growth factor activity of IL-6 (Helle *et al.*, 1988).

The epithelial and B-lymphoid cells were studied for effects of IL-6 on IRF-1 synthesis and DNA-binding activity, as well as for transcriptional effects of IL-6 on a regulatory sequence of the IRF-1 gene promoter. This TTTCCC-CGAAA sequence, named palindromic IFN-response element (pIRE), has been found responsible for activation of the IRF-1 gene by IFN-γ (Sims *et al.*, 1993). The function of the pIRE enhancer was considered similar to that of other IFN-γ-activated sequences (GAS; Decker *et al.*, 1991), in that pIRE binds the 91 kDa subunit of the IFN-inducible ISGF3 complex (Kanno *et al.*, 1993). This subunit binds by itself to GAS elements (Shuai *et al.*, 1992) and is now termed Stat91 (Sadowski *et al.*, 1993). We have analyzed the effects of IL-6 and IFN-γ on the activities of factors binding to pIRE sequences, in comparison with the known functions of GAS-type elements.

Results

Induction of IRF-1 mRNA and protein by IL-6

Human breast carcinoma T47D cells treated for 1 h with recombinant human IL-6 showed an induction of IRF-1 mRNA similar to that obtained with human IFNs, either type I IFN-β or type II IFN-γ (Figure 1). The IRF-1 mRNA level was reduced again by 4 h, in line with the short half-life of this mRNA (Watanabe *et al.*, 1991). IL-6 also induced IRF-1 mRNA in the murine hybridoma B9 cells. Since IL-6 (10 U/ml) is required for B9 cell growth (Helle *et al.*, 1988), cells were first starved of IL-6 for 5 h. Readdition of IL-6 (100 U/ml) for 1 h resulted in high IRF-1 mRNA levels, as did addition of murine IFN-α,β or IFN-γ (Figure 1, lanes 10–12), in comparison with cells in which starvation of IL-6 was continued (lane 9). At 4 h after IL-6 readdition, the IRF-1 mRNA was no more increased than in starved cells. After prolonged (29 h) starvation for IL-6, the non-growing B9 cells had higher IRF-1 mRNA (lane 15) than cultures refed with high dose IL-6 for 24 h after 5 h of starvation (lane 16).

The IRF-1 protein detected by immunoblots in nuclear extracts from both cell types was increased following IL-6 addition (Figure 2). The level of IRF-1 protein at 1 h after IL-6 addition to T47D cells (lanes 7–10) was comparable to that induced by IFN-β or IFN-γ (lanes 11 and 16). The IRF-1 protein was still seen at 4 and 24 h after IL-6 treatment, but in reduced amounts (lanes 12–15). In B9 cells starved of IL-6 for 5 h, readdition of IL-6 increased the IRF-1 protein at 1 h (Figure 2, lanes 5 and 6). However, if the B9 cells were left without IL-6 for longer, a rise in IRF-1 protein was observed in these starved non-growing B9 cells (lanes 1–4), as seen above for the IRF-1 mRNA.

IRF-1 and IRF-2 DNA-binding in IL-6-treated cells

The IRF-1 DNA-binding activity in IL-6-treated cells was measured in DNA electrophoretic mobility shift assays (EMSAs) with the $(AAGTGA)_3$ probe C13, which specifically binds proteins of the IRF family such as IRF-1 and IRF-2 (Fujita *et al.*, 1987; Harada *et al.*, 1989) and ICSBP (Driggers *et al.*, 1990) but not the ISGF3 complex (Harroch *et al.*, 1993). In nuclear extracts of breast carcinoma T47D cells treated for 1 h with IL-6, IFN-β or IFN-γ (Figure 3A, lanes 1–8), there was increased formation of the C13 complex migrating as the one formed by IRF-1 translated in reticulocyte lysates (lane 17). Addition of antibodies to IRF-1 and IRF-2 confirmed that it is the IRF-1 complex which is induced by IL-6 as well as by IFNs (lanes 13–16) but not the IRF-2 complex (lanes 9–12). IRF-1 DNA-binding activity was still increased at 4 h after IL-6 (lanes 5 and 6), but not at 24 h (lanes 7 and 8).

In contrast, nuclear extracts of murine hybridoma B9 cells showed a marked upper complex migrating like the one formed by IRF-2 (Figure 3B, compare lanes 1–13 with lane 20). Antibodies confirmed that no IRF-1-binding activity was present in the B9 cells, with or without IL-6 or IFNs, all DNA binding being abolished by anti-IRF-2 (lanes 14–18). The IRF-2 complex in B9 cells was not significantly changed at 1 h after IL-6 or IFNs (lanes 1–4 and 7–9). At 24 h after IL-6 readdition, there was a reduction in IRF-2 binding (lanes 5, 6 and 11–13) but no IRF-1 appeared (lanes 17 and 18). Another experiment indicated that high IRF-2 DNA-binding activity in B9 cells is constitutive since it was present in cells continuously growing in 10 U/ml IL-6 (Figure 3C, lane 8), as well as in cells starved of IL-6 for 29 h (lane 6). Here again, the readdition of 100 U/ml IL-6 to starved cells decreased IRF-2-binding activity (lanes 2 and 7). Analysis of T47D extracts in the same EMSA (Figure 3C, lanes 9–16) confirmed induction of the IRF-1 complex by IL-6 or IFNs in this cell contrasting with its absence in the hybridoma cell.

In view of the predominant IRF-2 DNA-binding in B9 cells, we examined the IRF-2 mRNA levels of these cells. The basal level of IRF-2 mRNA was not affected by 1 h treatment with IL-6 or IFN (Figure 4) in contrast to the induction seen for IRF-1 mRNA (Figure 1). There was an increase in IRF-2 mRNA after prolonged starvation for IL-6 (Figure 4, lane 7 showing 29 h starvation). B9 cells refed by 100 U/ml IL-6 for 4–24 h had a down-regulation of IRF-2 mRNA as compared with the corresponding starved cells (compare lanes 6 and 8 with 5 and 7). These changes correlate with the IRF-2 DNA-binding activities (Figure 3B, compare lane 6 with lane 5).

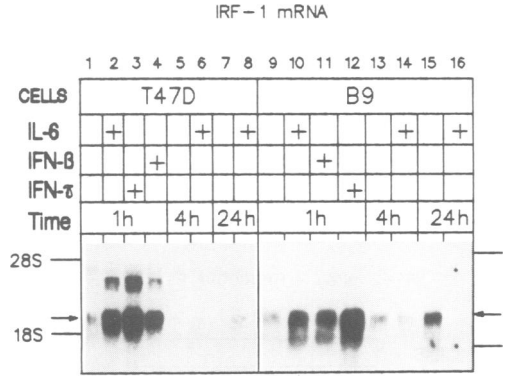

Fig. 1. Induction of IRF-1 mRNA by IL-6 and type I and II IFNs. Northern blot analysis of total cell RNA (20 μg/lane) from T47D and B9 cells treated for the indicated times with recombinant human IL-6 (100 U/ml) and for T47D treated with human IFN-β or IFN-γ, and for B9 treated with murine IFN-α,β or IFN-γ (500 U/ml). The B9 cells were starved of IL-6 for 5 h before treatment (see Materials and methods). The blots were reacted with either human or mouse IRF-1 cDNA radiolabeled probes. Hybridization with a probe for 18S rRNA and analysis of radioactivity in a Phospho-Imager (Fujix BAS1000) revealed no differences in the RNA loaded in each lane (not shown).

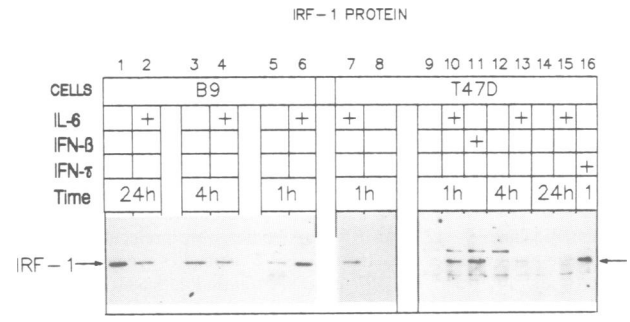

Fig. 2. Induction of IRF-1 protein by IL-6 and type I and II IFNs. Proteins from nuclear extracts of T47D and B9 cells (treated as in Figure 1), were analyzed in different Western electrophoretic blots with polyclonal anti-IRF-1 antibodies and ^{125}I-labeled protein A. Two experiments with T47D cells are shown (lanes 7 and 8, and 9–16). Size markers indicated IRF-1 is 48 kDa in mouse B9 cells and 56 kDa in human T47D cells.

IL-6 activates the palindromic IFN-responsive element of the IRF-1 gene

Since IL-6 and IFNs cause IRF-1 mRNA increases in T47D and B9 cells, we studied the effect of IL-6 on an IRF-1 gene control element known to respond to IFN. In the IRF-1 gene promoter, an inverted repeat GAAAN(N) sequence was shown to mediate induction by IFN-γ (Sims *et al.*, 1993). This element is called a palindromic IFN-responsive element (pIRE; Kanno *et al.*, 1993; see Table I). pIRE-binding proteins and expression of pIRE-reporter genes are activated by IFN-γ, IFN-α giving weaker and transient effects (Sims *et al.*, 1993; Kanno *et al.*, 1993). Figure 5 shows that IL-6 treatment of breast carcinoma T47D cells activated proteins forming specific complexes with pIRE DNA. Nuclear extracts of untreated T47D cells had no pIRE protein binding, but two types of complexes, designated pIRE-a and pIRE-b formed in response to IL-6 (Figure 5A, lanes 2 and 5). The pIRE-a complex forms a slowly migrating doublet, which appeared as early as 5 min following IL-6 addition and more strongly at 15 min. After 1−4 h with IL-6 the pIRE complexes became less abundant, but longer exposure of the EMSA gels demonstrated IL-6-dependent pIRE-a formation for 24 h (Figure 5B, lanes 5−8). IL-6-dependent induction of the faster migrating pIRE-b complex was seen at 5−15 min and decreased thereafter. The pIRE-b complex was the main complex formed when the T47D cells were treated by IFN-γ, reaching maximal levels at 1−4 h (Figure 5A, lanes 11 and 15). IFN-γ induced small amounts of pIRE-a, which were seen only in long exposures of the EMSA gels and always low compared to pIRE-b (Figure 5B, lane 4). IFN-β treatment produced a pattern intermediate between IL-6 and IFN-γ: pIRE-b was induced early but pIRE-a also appeared, even exceeding pIRE-b at 1 h after IFN-β (Figure 5A, lane 10; Figure 5B, lane 3). With IFN-β, pIRE-b was lower than with IFN-γ and also disappeared from 4 to 24 h, while remaining with IFN-γ (not shown).

The B9 hybridoma cells showed IL-6-dependent pIRE protein binding as well. Nuclear extracts of B9 cells growing in 10 U/ml IL-6 had pIRE-a activity which completely disappeared after removing IL-6 for 6 h but accumulated again upon addition of 100 U/ml IL-6 to the starved cells for 1−24 h (not shown).

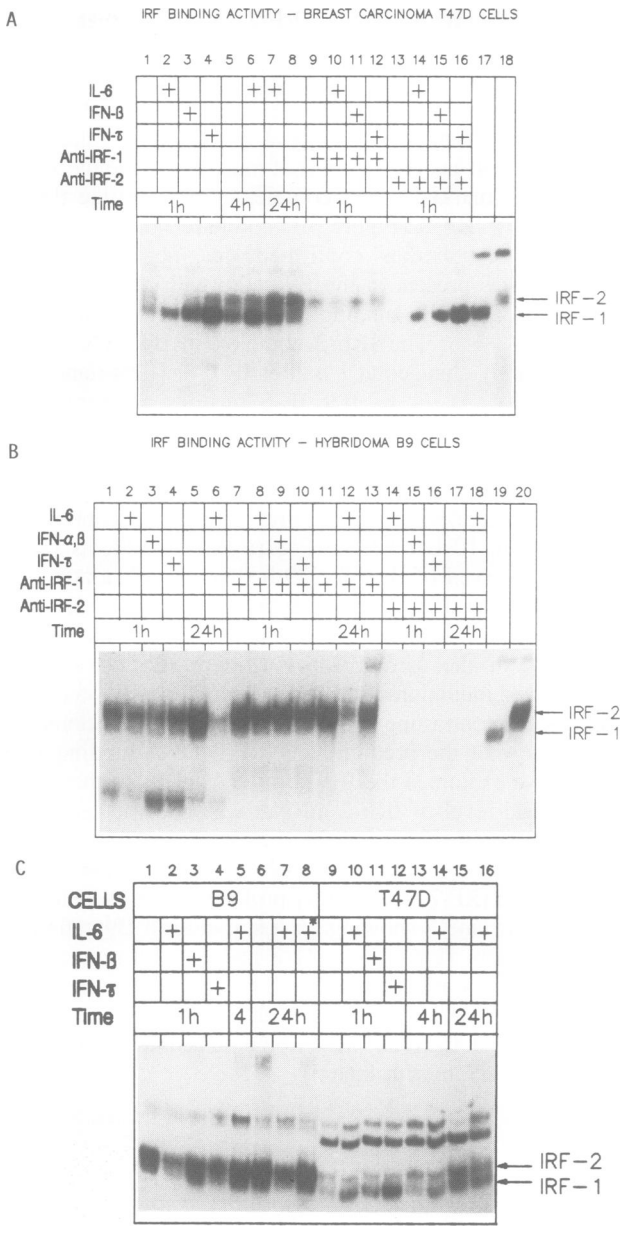

Fig. 3. DNA-binding activities of IRF-1 and IRF-2 in B9 and T47D cells. Nuclear extracts from the two cell types treated by the cytokines (as in Figure 1) were assayed by DNA EMSAs with the C13 (IRF-binding site) probe. (**A**) Lanes 1−16, T47D cell nuclear proteins (5 μg per lane). In lanes 9−12, anti-IRF-1 antibodies were preincubated with the extracts, and in lanes 13−16, anti-IRF-2 antibodies were used. Lanes 16 and 17, IRF-1 and IRF-2 cDNA translation products in reticulocyte lysates (1 μl per lane) were reacted with the C13 probe. (**B**) Lanes 1−18, B9 cell nuclear proteins (5 μg/lane). Anti-IRF-1 (lanes 7−13) and anti-IRF-2 (lanes 14−18) antibodies were preincubated with the extracts. Lanes 19 and 20, IRF-1 and IRF-2 translation products as in A. (**C**) Comparison on the same gel of the B9 and T47D nuclear extracts. Lane 8 (*) shows nuclear extracts from B9 cells grown for 29 h with 10 U/ml IL-6, compared with B9 cells (2×10^5/ml) kept for 29 h without IL-6 (lane 6) and with B9 cells kept for 5 h without IL-6 followed by addition of 100 U/ml IL-6 for 24 h (lane 7).

Different DNA sequence specificities of pIRE-a and pIRE-b

The pIRE-a and pIRE-b complexes differ not only in their cytokine-specific kinetics, but also in their affinity for different sequences evaluated with oligonucleotide competitors (Table I). Sequences in which the TT/AA at positions 2 and 3 in the palindrome were mutated (mutants 2 and 3 in Figure 5C, lanes 7, 8, 15 and 16) competed neither pIRE-a nor pIRE-b. Position 4 in the palindrome could be mutated

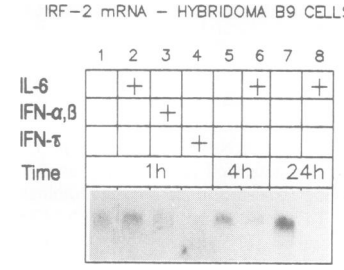

Fig. 4. Down-regulation of IRF-2 mRNA in B9 cells by IL-6. Total cell RNA from B9 cells, treated as in Figure 1, was assayed in Northern blots with a mouse IRF-2 cDNA probe.

Table I. pIRE sequences forming the pIRE-a complex

Competitors			Competitor affinity for pIRE-a complex
		5 4 3 2 1 – – – 1 2 3 4 5	
pIRE/IRF-1	5'-	C T G A T T T C C C C G A A A T G A C G G	++
	5'-	C C G T C A T T T C G G G G A A A T C A G	
ICSBP	5'-	G T G A T T T C **T** C **G** G A A A G A G A G	++
	5'-	C T C T C T T T C C G A G A A A T C A C	
MYD88	5'-	G A G **C** T T C **T** C **G** G A A A G C G A A A G A A G	++
	5'-	C T T C T T T C G C T T T C C G A G A A G C T C	
pIRE/FcR	5'-	C T G A T T T C C C **A** G A A A T G A C G G	++
	5'-	C C G T C A T T T C T G G G A A A T C A G	
pIRE/α_2M	5'-	C T G A T T T C **T** G **G** G A A A T G A C G G	++
	5'-	C C G T C A T T T C C C A G A A A T C A G	
Consensus		T T T C C C C G A A A	
		C T G G G	
		A	
		5 4 3 2 1 – – – 1 2 3 4 5	
GAS/GBP	5'-	A G T T T C A T **A** T T **A** C **T** C T A A A T C	–/+
	5'-	G A T T T A G A G T A A T A T G A A A C T	
Mutant 1	5'-	C T G A T T T C **G** C C G A A A T G A C G G	–/+
	5'-	C C G T C A T T T C G G C G A A A T C A G	
Mutant 2	5'-	C T G A T **A** A C C C C G A A A T G A C G G	–
	5'	C C G T C A T T T C G G G G T T A T C A G	
Mutant 3	5'-	C T G A T **A** T C C C C **C** A A A T G A C G G	–
	5'-	C C G T C A T T T G G G G G A T A T C A G	
CRP	5'-	C G G C A T **A G T G G C G C** A A A C T C C C T T A C T G	–
	5'-	C A G T A A G G G A G T T T G C G C C A C T A T G C C G	

Palindromic positions are numbered. pIRE/IRF-1 and ICSBP sequences from Sims *et al.* (1993) and Kanno *et al.* (1993). MYD88 sequence derived from an IL-6-induced gene (Lord *et al.*, 1990) whose promoter was sequenced in our laboratory. pIRE/FcR and pIRE/α_2M are the pIRE/IRF-1 with changes in the central spacer as in the human Fcγ receptor (Perez *et al.*, 1993) and rat α_2M genes (Wegenka *et al.*, 1993). GAS/GBP from Decker *et al.* (1991). CRP is an NF-IL-6-binding site (Oliviero and Cortese, 1989; Akira *et al.*, 1990). Deviations from pIRE/IRF-1 core are in bold.

without abolishing competition of pIRE-a, as shown by the Myd oligonucleotide (lanes 9 and 10). This sequence was derived from our study of the promoter of MYD88 (S.Harroch *et al.*, unpublished data), a gene inducible by IL-6 in M1 cells (Lord *et al.*, 1990). A differential affinity for pIRE-a and pIRE-b was seen with the GAS/GBP and mutant 1 sequences which competed pIRE-b more than pIRE-a (lanes 17 and 19). Competitor concentrations were determined in which GAS/GBP and mutant 1 competed pIRE-b by 80% (Table II). pIRE-a was not affected at all by GAS or mutant 1 under these conditions (Table II) whereas pIRE/IRF-1 competed both pIRE-a and b, mutant 3 competing neither of them. Comparison of the oligonucleotides which bind to pIRE-a allowed us to deduce a consensus sequence (Table I). GAS/GBP differs from the consensus in position 1 of the palindrome and in the middle base pair of the central spacer. Mutant 1 differs only in the first base of the spacer (G instead of Py). Such changes appear to affect binding to the pIRE-a factors.

Activation of pIRE and GAS reporter genes by IL-6 and IFN-γ

The DNA-binding data indicated that pIRE-a and pIRE-b factors are not functionally equivalent for pIRE and GAS elements. The stronger induction of pIRE-a by IL-6 and of pIRE-b relatively by IFN-γ, might then affect how these cytokines control genes harboring GAS or pIRE enhancers. This suggestion was verified by reporter gene expression.

In transfection assays with T47D cells, IL-6 activated several hundred-fold the expression of luciferase reporter gene (pGL2) constructs containing oligomers of the pIRE/IRF-1 or pIRE/α_2M (Table III). Treatments of 10–14 h with IL-6 or IFN-γ gave maximal luciferase induction, IFN-β having smaller and more transient effects. Mutant 2 sequence which does not bind pIRE factors, gave negligible luciferase induction (Table III). The relative activities of IL-6 and IFN-γ depended on the enhancer DNA sequence. Whereas IL-6 could elicit higher luciferase inductions than IFN-γ on pIRE/α_2M and pIRE/IRF-1 constructs, the response of GAS/GBP constructs to IL-6 was 11–18% of that of IFN-γ (Table III). The different ratio of IL-6 and IFN-γ responses with pIRE and GAS reporter genes shows that the regulation of these two related transcriptional elements is not identical, unlike what had been assumed (Kanno *et al.*, 1993).

pIRE-b but not pIRE-a contains the Stat91 protein

The pIRE complex induced by IFN-γ in T-lymphoid EL4 cells, was shown to contain Stat91 (Kanno *et al.*, 1993). We tested the ability of rabbit anti-Stat91 antibodies to perturb the formation of pIRE-a and pIRE-b complexes. In extracts of T47D mammary cells stimulated by IL-6, the pIRE-b complex disappeared with antibodies to Stat91 but pIRE-a was unaffected as compared with control antibodies (Figure 6). The pIRE-b complex induced by IFN-γ disappeared with anti-Stat91 like that induced by IL-6, both being possibly supershifted (Figure 6). Different factor(s)

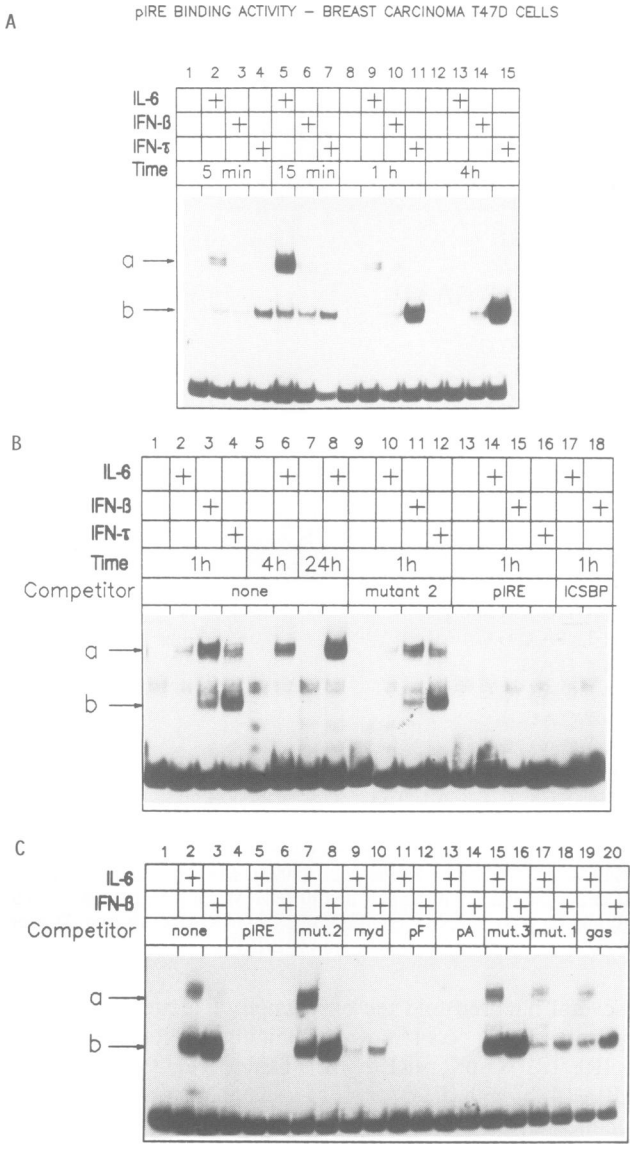

Fig. 5. IL-6, IFN-β and IFN-γ activate the binding of distinct factors to the pIRE element of the IRF-1 gene in T47D cells. Nuclear extracts of T47D cells treated by cytokines (as in Figure 1) were subjected to DNA EMSAs with the pIRE/IRF-1 probe (Table I). (**A**) Short-term kinetics of activation. Autoradiography with Agfa Curix A films for 24 h. Position of pIRE-a and pIRE-b factor complexes is indicated by arrows. (**B**) Long-term kinetics of activation. Autoradiographic exposure of 3 days. The competitor oligonucleotides (see Table I), mutant 2 (lanes 9–12), pIRE/IRF-1 (lanes 13–16) and ICSBP (lanes 7 and 18) were added at a 100-fold molar excess over the labeled pIRE/IRF-1 probe. (**C**) Nuclear extracts of T47D cells treated for 5 min with IL-6 or with IFN-β were assayed with the pIRE/IRF-1 probe and the following competitors (see Table I): pIRE/IRF-1 (lanes 4–6), mutant 2 (lanes 7 and 8), MYD88 (lanes 9 and 10), pIRE/FcR (lanes 11 and 12), pIRE/α_2M (lanes 13 and 14), mutant 1 (lanes 17 and 18) and GAS/GBP (lanes 19 and 20).

than the known Stat91 protein appear to be involved in forming the pIRE-a complex characteristic of IL-6 action.

Discussion

IL-6 activates the pIRE promoter sequence of the IRF-1 gene

Induced expression of the IRF-1 transcription factor is an early response to IL-6 in different cell types. IL-6 acts on the palindromic transcriptional regulatory pIRE sequence of the IRF-1 gene promoter, known to mediate activation by IFN-γ and more weakly by IFN-α (Kanno *et al.*, 1993; Sims *et al.*, 1993). The pIRE sequence is highly responsive to IL-6 when tested in reporter gene transfections of the breast carcinoma cells. Within 5 min of IL-6 addition to these cells, two distinct pIRE–protein complexes are induced which differ in protein composition and functional significance.

Table II. Effect of competitors on pIRE-a and pIRE-b complexes

Competitor	Residual complex (%)	
	pIRE-a	pIRE-b
None	100	100
pIRE/IRF-1	8	0
GAS/GBP	101	21
Mutant 1	103	20
Mutant 3	145	105

Nuclear extracts from T47D cells treated for 5 min with IL-6 were used in DNA EMSAs with the pIRE/IRF-1 probe with competitor probes shown in Table I. The ratio of competitor to labeled probe was determined in preliminary experiments to give at leat 80% competition of the pIRE-b complex. The amount of IL-6-induced complex was quantified in a Phospho-Imager (Fujix BAS1000).

The rapidly migrating pIRE-b complex corresponds to the main complex formed with pIRE sequences in response to IFN-γ. In line with studies on IFN-γ (Kanno *et al.*, 1993), the IL-6-induced pIRE-b complex contains the Stat91 protein and is competed by IFN-γ-responsive DNA sequences such as GAS/GBP. In response to IFN-γ, Stat91 binds by itself to GAS/GBP (Shuai *et al.*, 1992) or to similar elements such as the GIRE in the Fcγ receptor gene (Perez *et al.*, 1993), and the serum-inducible element (SIE) in the c-*fos* gene (Sadowski *et al.*, 1993). IFN-γ induces Stat91 tyrosine phosphorylation (Shuai *et al.*, 1992) and tyrosine kinases Jak1 and Jak2 have been implicated in IFN-γ action (Muller *et al.*, 1993). Various growth factors and cytokines can activate Stat91 binding to GAS- or SIE-type elements (Larner *et al.*, 1993; Sadowski *et al.*, 1993; Silvennoinen *et al.*, 1993) and IL-6 has clearly such effects on Stat91.

The predominant complex induced by IL-6 is the slowly migrating pIRE-a which does not contain Stat91. The pIRE-a factor(s) has lower affinity for GAS elements than for the IRF-1 gene pIRE sequence or consensus pIRE. Functional differences of pIRE and GAS elements are revealed by transfection experiments in the breast carcinoma cells. Thus, transcriptional activation by IL-6 relative to IFN-γ is higher for pIRE- than for GAS-driven genes. The observed difference correlates with the properties of the pIRE-a and pIRE-b complexes and their contrasting ratios of induction by IL-6 and IFN-γ. This differential behavior of the two palindromic enhancers helps understand how various cytokines and growth factors can activate specific gene patterns, beyond their common action through Stat91 on pIRE, GAS or SIE control elements (Larner *et al.*, 1993; Sadowski *et al.*, 1993; Silvennoinen *et al.*, 1993). Distinct transcription complexes forming in ratios dependent on the cytokine signal, can lead to a specific spectrum of action on various genes controlled by these related elements.

IFN-β also produces higher pIRE-a:pIRE-b ratios than does IFN-γ. A slowly migrating pIRE complex was observed

Table III. Expression of pIRE–luciferase in IL-6-treated T47D cells

Sequence	Time (h)	Luciferase Fold induction IL-6	IFN-β	IFN-γ	Ratio IL-6/IFN-γ
pIRE/α_2M	3	88.6	29.8	61.0	1.45
	5	107.1	23.5	72.5	1.48
	10	194.6	10.5	114.6	1.70
	14	267.7	4.3	94.0	2.85
	30	55.0	1.6	18.8	2.92
pIRE/IRF-1	10	61.4	13.1	101.7	0.60
	14	184.4	7.4	132.0	1.40
GAS/GBP	10	7.1	15.4	62.9	0.11
	14	17.8	14.9	97.0	0.18
Mutant 2	10	2.8	1.1	1.0	
	14	4.4	1.4	1.9	

T47D cells were transfected in suspension with luciferase gene constructs fused to the indicated pIRE or GAS sequences (see Table I and Materials and methods). The cells were then plated in 3.5 cm wells and, 26 h post-transfection, were treated with 100 U/ml IL-6, 500 U/ml IFN-γ or IFN-β, or left untreated. After the indicated times, cell extracts were assayed for luciferase activity with cytokine as compared to without cytokine. Expression of the internal β-galactosidase control plasmid was similar in all conditions (not shown).

with IFN-α,β, but not with IFN-γ, in T-lymphoid cells (Kanno *et al.*, 1993). IFN-α,β action involves Jak1 and Tyk2 kinases (Muller *et al.*, 1993) and tyrosine phosphorylation of both Stat91 and p113 factors (Fu *et al.*, 1992; Schindler *et al.*, 1992) which associate with IRF-related p48 (Veals *et al.*, 1992) to form ISGF3 on ISRE sites of IFN-activatable genes. Stat91 activation by IFN-β can account for the pIRE-b complex, but IFN-β must have yet other effects leading to pIRE-a formation. With either IL-6 or IFN-β, pIRE-a is formed by tyrosine phosphorylated factors different from Stat91 (S.Harroch *et al.*, submitted).

The rapid activation of pIRE-binding proteins provides a system to study signal transduction by IL-6 in a variety of cell types. Previously, studies in liver cells indicated the role of NF-IL-6 in IL-6 induction of acute phase protein (APP) genes (Akira *et al.*, 1990; Poli *et al.*, 1990; Natsuka *et al.*, 1991). Competition with NF-IL-6-binding sites (e.g. CRP in Table I) failed to affect pIRE protein-binding. Another IL-6-induced liver APP gene factor is APRF, described by Wegenka *et al.* (1993) as recognizing 'CTGGGA' elements including TTCTGGGAA (similar to pIRE), but also TAACTGGAA which would not conform to the pIRE-a consensus. SIE sequences also form an IL-6-induced complex with a liver APRF activity which is not Stat91 (Sadowski *et al.*, 1993), making it unlikely that APRF has the same specificity as the pIRE-a factor from breast carcinoma cells. In liver, IL-6 activates yet another factor related to Ets and implicated in JunB activation (Nakajima *et al.*, 1993). The pIRE system now allows the mechanisms by which IL-6 activates these various transcription factors in the liver and in other cell types to be compared.

Cell type-dependent control of IRF-1 activity

The effects of IL-6 on the IRF-1 gene regulatory element correlate with the increases in IRF-1 mRNA and protein seen in the T47D breast carcinoma and B9 hybridoma cells. However, only T47D showed induced IRF-1 DNA-binding activity. Nuclear extracts of B9 cells, containing IL-6-induced IRF-1 protein, had no IRF-1 DNA-binding activity but instead constitutive IRF-2 binding. We previously

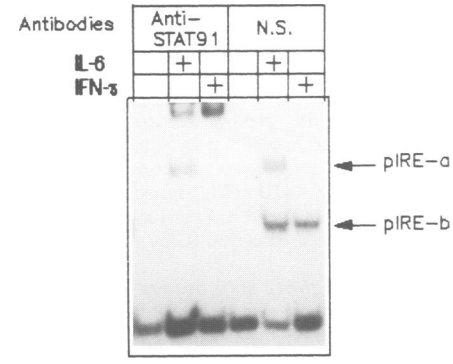

Fig. 6. Effect of antibodies to Stat91 on pIRE-a and pIRE-b complexes. Nuclear extracts from T47D cells treated for 5 min with IL-6 or IFN-γ (as in Figure 1) were pre-treated with antibodies to Stat91-84 or unrelated rabbit antiserum (N.S., both sera diluted 1:5), before DNA EMSA with the pIRE/IRF-1 probe.

observed that IL-6 induces IRF-1 DNA-binding in M1 cells responding to IL-6 by growth arrest and differentiation, but not in M1 mutants which grow in the presence of IL-6 (Harroch *et al.*, 1993). These resistant M1 cells also had high constitutive IRF-2-binding activity. In several systems, high IRF-2 appears to have growth-stimulatory action and IRF-1 to have growth-inhibitory effects (Harada *et al.*, 1993; Kirchhoff *et al.*, 1993). The difference between IRF-1 and IRF-2 DNA-binding activities in T47D cells which are growth-inhibited by IL-6 (Novick *et al.*, 1992) and B9 cells which are growth-stimulated (Helle *et al.*, 1988), would be in line with such roles of IRF-1 and IRF-2 in cell proliferation. This relation is further supported by the fact that the B9 cells had lower IRF-2 binding at 100 than at 10 U/ml IL-6, cell proliferation being also much lower at the high IL-6 dose than at the lower one (not shown). The level of IL-6-induced IRF-1-binding activity relative to constitutive IRF-2 binding may serve as an indicator of the growth regulations exerted by IL-6 and IFNs.

Comparisons of IFNs and IL-6 reveal similarities in their effects on IRL-1 gene pIRE control elements and on

accumulation of active IRF-1 protein. From studies on M1 cells (Cohen *et al.*, 1991; Harroch *et al.*, 1993), we proposed that IFN-like effects of IL-6 [e.g. (2′-5′) A synthetase, MHC inductions] could be explained by IRF-1-enhancing effects of autocrine IFN since IRF-1 increases responses to IFNs (Reis *et al.*, 1992) or mimics IFN action (Pine, 1992). By activating Stat91 (pIRE-b), IL-6 may also act synergistically with IFN-α,β through ISGF3 as does IFN-γ. The regulation of the IRF-1 gene and protein activities provides a convenient model to study interactions between IFNs and IL-6, as well as other cytokines with effects on IRF-1 such as IL-1 and TNF (Fujita *et al.*, 1989; Watanabe *et al.*, 1991).

Materials and methods

Cell lines and cytokines

The human breast carcinoma T47D clone 07 cells (Chen *et al.*, 1991) were cultured as monolayers in RPMI 1640, 10% fetal calf serum (FCS) and 10 μg/ml human insulin (Novo Nordisk), at 37°C in 5% CO_2. The T47D cells were subcultured 1 day before stimulation by cytokines. The murine hybridoma B9 cells (Helle *et al.*, 1988) were grown to 10^6 cells/ml in suspension with RPMI 1640 (Biolabs, Israel), 10% heat-inactivated FCS and 10 U/ml recombinant human IL-6. Before use, B9 cells were washed and resuspended at 2×10^5 cells/ml of fresh medium without IL-6, and further cultured for 5 h before stimulation by cytokines.

Recombinant human IL-6 (5×10^6 U/mg, <0.1 ng endotoxin/mg), prepared as described from Chinese hamster ovary (CHO) cells and titrated on T1165 plasmacytoma cells (Novick *et al.*, 1989), was obtained from InterPharm Laboratories (IPL, Nes-Ziona, Israel) and used at 20 ng/ml (100 U/ml). Recombinant human IFN-β (5×10^8 IU/mg) and IFN-γ (10^8 IU/mg) from CHO cells (IPL) were as described by Chen *et al.* (1991) and used at 500 IU/ml on T47D cells. Murine IFN-α,β was from Lee Biomolecular Research (San Diego, CA) and recombinant murine IFN-γ from Genzyme; both were used at 500 IU/ml on B9 cells.

DNA electrophoretic mobility shift assays

Treated cells were washed twice in ice-cold phosphate buffered saline (PBS), and pellets frozen in liquid N_2. After thawing in 4 vols of Buffer W [10 mM HEPES pH 7.9, 0.1 mM EDTA, 10 mM NaCl, 1 mM dithiothreitol, 5% (v/v) glycerol, 50 mM NaF, 0.1 mM sodium vanadate, 10 mM sodium molybdate, 0.5 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, 4 μg/ml aprotinin, 2 μg/ml chymostatin, 1.5 μg/ml pepstatin, 2 μg/ml antipain], and repeated pipetting, the lysate was centrifuged at 5000 r.p.m. for 10 min (Eppendorf microfuge). The pellets were resuspended in 2.5 vols of Buffer W containing 0.4 M NaCl and recentrifuged at 14 000 r.p.m. for 15 min, and the supernatant was used as nuclear extract.

The C13 probe ctagAAGTGAAAGTGAAAGTGA was prepared and used as described by Harroch *et al.* (1993). The pIRE/IRF-1 probe was formed by annealing 5′-gatcCTGATTTCCCCGAAATGACGG-3′ with 3′-GACTAAAGGGGCTTTCATGCCgatc-5′, and end-labeling by [γ-^{32}P]ATP with T4 polynucleotide kinase, to 2×10^3 c.p.m./fmol. Competitors used are shown in Table I. For EMSA, equal amounts of nuclear extract proteins (5–10 μg) were mixed with 2×10^4 c.p.m. of DNA probe, 2.5–5 μg poly(dI)(dC) (Pharmacia), with or without cold competitors (100-fold molar excess over labeled probe), in a final volume of 20 μl containing 20 mM Tris–HCl pH 7.9, 50 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 5 mM dithiothreitol, 5 mM $MgCl_2$, 1 μg salmon sperm DNA and 1 mM spermidine. When used, antibodies (see below) were added as 1 μl of rabbit control or immune sera to 5 μg protein in 5 μl with 3× above salts and buffer for 30 min at 0°C, before adding the labeled probe, with 1 μg salmon sperm DNA and 2.5 μg poly(dI-dC) in a final volume of 20 μl as above. After 15 min at 25°C, electrophoresis was carried out in 5% acrylamide/bisacrylamide (38:2) gels with 25 mM Tris–borate pH 8.2, 0.5 mM EDTA for 2.5–3 h at 175 V. Gels were dried and subjected to autoradiography.

Northern blot analysis

Total cell RNA was extracted from 4×10^7 B9 cells, or from 9 cm plates of T47D cells using the TRI Reagent (Molecular Research Center). RNA was electrophoresed on denaturating formaldehyde–agarose gels, blotted onto GeneScreen Plus (Dupont, DE) and reacted with cDNA probes labeled with [^{32}P]dCTP by random priming (Boehringer, Mannheim). The murine IRF-1 cDNA probe was the 1 kb *Eco*RI fragment from pBHIRF1, gift of Dr H.Hauser, GBF, Braunschweig (Kirchhoff *et al.*, 1993). The human IRF-1 probe was the *Eco*RV–*Bgl*II fragment of the pUChIRF1 plasmid and the IRF-2 probe was the *Eco*RI–*Eco*RV fragment of plasmid pIRF2-5, obtained from Dr H.Harada, Osaka University (Harada *et al.*, 1989).

Antibodies

A *Bam*HI–*Eco*RI fragment of the pGEM-2/IRF-1 plasmid (gift of Dr H.Hauser) containing the full-length coding sequence of murine IRF-1, was inserted in the expression vector pGEX-3X (Pharmacia). The GST–IRF-1 fusion protein, produced in *E.coli*, purified on glutathione columns or extracted from SDS–polyacrylamide gels, was injected into rabbits. After the fourth booster injection, immunoglobulin from rabbit sera were prepared by ammonium sulfate precipitation and dialyzed against PBS. The specificity of the antibodies was verified by immunoprecipitation of [^{35}S]methionine-labeled IRF-1 translated *in vitro* in reticulocyte lysates, as well as by binding to the recombinant protein in Western blots. Antibodies to Stat91 were produced by the above method using a *Sma*I–*Xba*I cDNA fragment cut from PCR products made with primers (coordinates 1628–1646 and 2364–2384) from the published sequence (Fu *et al.*, 1992). Antibodies to murine recombinant IRF-2 (Harada *et al.*, 1990) were a kind gift from Drs N.Watanabe and T.Taniguchi (Osaka).

Western blot analysis

Proteins (20 μg) from nuclear extracts were analyzed by 10%-polyacrylamide gel electrophoresis in SDS, and electroblotted to nitrocellulose. Blocking of non-specific binding, was done for 2 h in PBST (0.05% Tween 20 in PBS) with 10% (w/v) dried low-fat milk. Anti-IRF-1 antibodies, diluted 1:200 in PBST with 5% milk, were reacted for 5 h at room temperature, and ^{125}I-labeled protein A (Amersham, UK) was used for detection.

Transfection and luciferase assays

The double-stranded oligonucleotides pIRE/IRF-1, pIRE/α_2M, GAS/GBP and mutant 3 (Table I), synthesized with 5′ protruding GATC ends, were polymerized with T4 DNA ligase and size-selected oligomers (four of five repeats) were cloned into the *Bgl*II site of the pGL2-pv vector (Promega), upstream of the SV40 early promoter and luciferase gene. For transfection, 4×10^7 T47D cells were trypsinized, washed once in PBS and suspended in 10 ml TD buffer (25 mM Tris–HCl pH 7.4, 140 mM NaCl, 5 mM KCl, 0.7 mM K_2HPO_4) supplemented with 100 μg of the above plasmid constructs together with 20 μg pβ-GAL plasmid (Promega) as internal control and 6 mg of DEAE–dextran for 20 min at room temperature. The cells were then washed twice and plated in multiwell tissue culture plates (2×10^6 cells/3.5 cm well/3 ml culture medium). After 26 h, IL-6, IFN-β or IFN-γ were added to cells of the same transfection, which at indicated times were assayed with the extraction (1% Triton X-100, 0.25 ml/well) and luciferase assay kit of Promega.

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Note added in proof

The ICSBP sequence which competes pIRE-a does not form pIRE-a when used itself as a probe in mobility shift assays, indicating further specificity of the pIRE-a factors.