1. Introduction

The interferon regulatory factor (IRF) family of transcription factors (IRF-1 to IRF-10) controls virus infection, host response to pathogens, innate and adaptive immunity, cell growth and proliferation, differentiation of cell types during development and functional specialization of the immune system in mammals (Taniguchi et al. 2001). IRFs control expression of cytokines, growth factors, cell adhesion molecules, chemokines and regulate a large number of genes in a variety of cells and tissues under diverse conditions (Ozato et al. 2007). Dysregulation of IRFs by gene mutation (Nishio et al. 2001), differential expression (Choo et al. 2006), alternative splicing of the mRNAs (Maratheftis et al. 2006), post-translational modification and proteolytic processing (Park et al. 2007) during various pathological conditions and diseases have been reported. IRF-1 and IRF-2, the two founding members of IRF family, provide examples of both positive and negative regulators of transcription of genes during immune response (Colonna 2007), cell growth (Masumi et al. 2003), differentiation (Chung and Kawamoto 2004) and cancer (Wang et al. 2007). For example, IRF-1 induces expression of interferon (IFN) (Honda et al. 2006), inducible

Keywords. Conformation; DNA-binding; IRF-2; tetrapeptide

Abbreviations used: CPM, counts per minute; GST, glutathione s-transferase; IPTG, isopropyl thiogalactoside; PBS, phosphate buffered saline; pBS, plasmid Bluescript; PCR, polymerase chain reaction; SDS-PAGE, sodium dodesyl sulfate-polyacrylamide gel electrophoresis
nitric oxide synthase (iNOS) (Spink and Evans 1997), cyclooxygenase-2 (Cox-2) (Blanco et al. 2000), vascular cell adhesion molecule-1 (VCAM-1) (Neish et al. 1995), interleukin-12 (IL-12) (Kollet et al. 2001), caspase 1 (interleukin converting enzyme or ICE) (Jain et al. 2005) genes, whereas IRF-2 induces expression of histone H4 (Xie et al. 2001), interleukin-7 (IL-7) (Oshima et al. 2004), transporter of antigenic peptides to MHC class I (TAP1) (Rouyez et al. 2005), Fas ligand (Fas L) (Chow et al. 2000) and tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) (Clarke et al. 2004) genes. IRF-1 and IRF-2 have evolved as “twin-transcription factors” to coordinate target gene expression with cell signalling events. IRF-1 has a C-terminal transcriptional activation domain (TAD) and IRF-2 has a C-terminal transcriptional repression domain (TRD), and both have a similar N-terminal DNA-binding domains (DBDs) (Harada et al. 1989). Their N-terminal DNA-binding regions have considerable homology (62%), but the C-terminal transcriptional activation/repression domains have lesser homology (25%) and greater diversity. Usually, IRF-1 is an activator and IRF-2, a repressor of IRF-1-mediated transcription, but IRF-2 by itself can also act as an activator (Masumi et al. 2006).

IRF-2 can suppress IRF-1-mediated gene expression through at least three mechanisms: (i) IRF-2 binds IRF-1-binding sites in the promoters/enhancers of IRF-1-target genes with greater affinity, (ii) IRF-1 up-regulates transcription of IRF-2 gene and (iii) IRF-1 protein has a half-life of only 30 min whereas IRF-2 protein has a half-life of 8 h. Genes regulated by IRF-1 and IRF-2 can be broadly classified into three categories on the basis of cellular responses: (a) IFN, other cytokines and antiviral proteins, (b) inflammation, immune response and differentiation of immune cells, and (c) cell growth/proliferation, apoptosis and tumorigenesis. IRF-1/IRF-2 target genes can also be divided into four categories based on the actions of IRF-1 and IRF-2: (i) genes induced by IRF-1, (ii) genes induced by IRF-1 and suppressed by IRF-2, (iii) genes induced by IRF-2 and (iv) genes cooperatively induced by IRF-1 and IRF-2. This complex network of gene regulation by IRF-1/IRF-2 is possible because of various domains, motifs and sites present in IRF-1 and IRF-2 proteins and causes a range of DNA binding and protein–protein interactions resulting in the plasticity of gene expression. Many functional regions in IRF-1 have been identified and characterized, but relatively fewer regions have been investigated in IRF-2. For example, the mechanisms of how IRF-2 can act as a repressor, a suppressor and an activator still remains to be fully understood. We hypothesized that IRF-2 might have evolved to possess a diversity of small motifs functioning in a variety of interdependent ways to bring about alterations in its DNA-binding and transcriptional activities. In order to test this, we compared mouse and human IRF-2 amino acid sequences and identified a C-terminal tetrapeptide at 314-317 sequence to be different in the two species: 314PAPV317 in mouse IRF-2 and 314SSSM317 in human IRF-2. We demonstrate that recombinant mouse IRF-2 (GST-IRF-2) proteins with 314PAPV317 (wild type) and 314SSSM317 (mutant) show similar protein expression and immunoreactivity but different N-terminal DNA-binding activities with 32P(GAAAGT)4 IRF-element. The DNA–protein complexes formed by the wt IRF-2 and mt IRF-2 are qualitatively and quantitatively different, possibly because of conformational variations, which get resolved after stabilization by anti-IRF-2 antibody.

2. Materials and methods

2.1 Plasmids, Escherichia coli cells, oligonucleotides, antibodies, radioisotope and reagents

The following were used for the study: mouse IRF-2 cDNA (pIRF2.5 plasmid), pBluescript II (pBS) cloning vector, pGEX2TK expression plasmid, E. coli DH5α- and XL-1 Blue cells, oligonucleotides (Sigma Chemicals Co., USA) for IRF-2 expression: 5′-coding and 3′-noncoding wild type, internal (314-317) coding and noncoding mutant primers; IRF-element (IRF-E) for DNA-binding assay: top strand (5′-GAAAGTGAAAGTGAAAGTGAAAGTGAAGATGTAG-3′) and bottom strand (5′-ACTTTCACTTTCACTTTCACTTTCACTTT-3′), anti-GST (G7781) and anti-rabbit IgG-HRP (A9169) antibodies (Sigma Chemicals Co., USA), anti-IRF-2 antibody (H-229: sc-13042, Santa Cruz Biotech, USA), [32P]ATP (specific activity = 4000 Ci/mmmole; BRIT, India), Pfu DNA polymerase (Stratagene), restriction enzymes, T4 DNA ligase and T4 polynucleotide kinase (New England Biolabs, MBI Fermentas) and molecular biology grade reagents (Sigma Chemicals Co.). The most commonly used molecular biology methods were adopted from the reference (Sambrook and Russell 2001) and suitably modified.

2.2 Recombinant wild type and mutant IRF-2

Protein coding sequence (1-349 a.a.) of mouse IRF-2 cDNA was PCR-amplified as a 1049 bp DNA from pIRF2.5 plasmid by amplification of the whole plasmid by circular PCR using IRF-2 primers (5′-coding: 5′AAGGATCCGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA...
C-terminal tetrapeptide influences DNA binding of IRF-2

from wt IRF-2 (5997 bp) by using internal mutant primers (IRF-2M1: 5′CCATTTACAGACCTTCCCCTTCCCTCC TCCAGACCCCAAGCCACGAGC3′ and IRF-2M2: 5′GCTGCTGGGCACTGGGGTCATGAGGAGGAAAG GGGAAGGTCTGTAAATGG 3′) containing 12 nt. mutant-sequence in the middle to alter 4 a.a. at 314-317 (PAPV was replaced by SSSM). The wild type PCR-DNA was digested by DpnI before transforming E. coli DH5α cells. Wt and mt GST-IRF-2 fusion proteins were expressed in E. coli XL-1 blue cells by 0.5 mM IPTG-induction at 37°C and the cell extracts were analysed by 10% SDS-PAGE and stained by Coomassie brilliant blue. The IPTG-induced/uninduced culture was centrifuged at 5k rpm for 5 min at 4°C; the cell pellet was washed with 5 ml PBS and resuspended in 5 ml of lysis buffer (PBS with 1% Triton X-100, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.5 μg/ml benzamidine), sonicated on ice at 15 μm amplitude for 15 s (six times with an interval of 1 min). The extract was clarified by centrifugation at 12k rpm for 5 min at 4°C. The supernatant was stored at −80°C and used for SDS-PAGE and immunoblotting.

2.3 Western blot

IPTG-induced wt- and mt IRF-2 cell extracts were resolved by 10% SDS-PAGE, electroblotted to nitro-cellulose filters, blocked by 5% milk in PBST (PBS with Tween-20), washed by PBST and incubated with anti-GST (0.2 μg/μl) (1:2000) or anti-IRF-2 (0.2 μg/μl) (1:1500) diluted antibody, washed by PBST, further incubated with anti-rabbit IgG-HRP secondary antibody (1:3000), washed by PBST and developed by DAB (3, 3′-diaminobenzidine)-staining.

2.4 Electrophoretic mobility shift assay (EMSA)

IPTG-induced wt- and mt GST-IRF-2 cell extracts (0.5–2.0 μg) prepared in EMSA-cell lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA (pH 8.0), 0.1 mM EGTA (pH 7.0), 1 mM DTT, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.5 μg/ml benzamidine] were incubated with 50 fmole of 5′[32P]-labelled double-stranded IRF-E (specific activity = 2×10⁶–4×10⁶ CPM/pmol) in EMSA-binding buffer [20 mM HEPES (pH 7.9), 0.4 mM EDTA (pH 8.0), 0.4 mM DTT, 5% glycerol] in 20 μl reactions at 37°C (or on ice) for 30 min, mixed with 4 μl of loading dye (glycerol and bromophenol blue) and electrophoresed in 7.5% native PAGE in EMSA-gel buffer [50 mM Tris, 400 mM Glycine, 2 mM EDTA (pH 8.5)] at 150 V (40 mA) to separate the DNA–protein complexes from free oligonucleotides. The gel was dried and exposed to phosphoimager screen, and the complexes were quantified as %pixel of free oligo. Unlabelled (50- or 100-fold) molar excess of IRF-E was used for competition and anti-IRF-2 antibody (0.2 μg) was used for DNA binding in the presence of antibody.

3. Results and discussion

3.1 Amino acid changes between mouse and human IRF-2

Table 1 and figure 1A show that most of the amino acid variations between mouse and human IRF-2 are in the transcriptional activation domain (TAD), IRF-association domain (IAD) and repression domain (RD), whereas the DNA-binding domain (DBD) remains largely conserved.

Table 1. Amino acid variations between murine IRF-2 and human IRF-2

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Amino acid residue no.</th>
<th>Mouse</th>
<th>Human</th>
<th>Region of IRF-2</th>
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<tr>
<td>1.</td>
<td>69</td>
<td>I</td>
<td>V</td>
<td>DBD</td>
</tr>
<tr>
<td>2.</td>
<td>97</td>
<td>R</td>
<td>K</td>
<td>DBD</td>
</tr>
<tr>
<td>3.</td>
<td>131</td>
<td>E</td>
<td>D</td>
<td>DBD-TAD</td>
</tr>
<tr>
<td>4.</td>
<td>132</td>
<td>R</td>
<td>K</td>
<td>DBD-TAD</td>
</tr>
<tr>
<td>5.</td>
<td>153</td>
<td>G</td>
<td>D</td>
<td>DBD-TAD</td>
</tr>
<tr>
<td>6.</td>
<td>154</td>
<td>F</td>
<td>L</td>
<td>DBD-TAD</td>
</tr>
<tr>
<td>7.</td>
<td>164</td>
<td>A</td>
<td>T</td>
<td>DBD-TAD</td>
</tr>
<tr>
<td>8.</td>
<td>189</td>
<td>D</td>
<td>N</td>
<td>TAD</td>
</tr>
<tr>
<td>9.</td>
<td>211</td>
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<td>E</td>
<td>TAD</td>
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<tr>
<td>10.</td>
<td>240</td>
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<td>P</td>
<td>IAD2</td>
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<tr>
<td>11.</td>
<td>245</td>
<td>N</td>
<td>S</td>
<td>IAD2</td>
</tr>
<tr>
<td>12.</td>
<td>256</td>
<td>S</td>
<td>N</td>
<td>IAD2</td>
</tr>
<tr>
<td>13.</td>
<td>270</td>
<td>N</td>
<td>G</td>
<td>IAD2-RD</td>
</tr>
<tr>
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<td>S</td>
<td>IAD2-RD</td>
</tr>
<tr>
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<td>G</td>
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<tr>
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</tr>
<tr>
<td>17.</td>
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<td>N</td>
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</tr>
<tr>
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<td>M</td>
<td>V</td>
<td>IAD2-RD</td>
</tr>
<tr>
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<td>IAD2-RD</td>
</tr>
<tr>
<td>20.</td>
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<td>P</td>
<td>S</td>
<td>IAD2-RD</td>
</tr>
<tr>
<td>21.</td>
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<td>A</td>
<td>S</td>
<td>IAD2-RD</td>
</tr>
<tr>
<td>22.</td>
<td>316</td>
<td>P</td>
<td>S</td>
<td>IAD2-RD</td>
</tr>
<tr>
<td>23.</td>
<td>317</td>
<td>V</td>
<td>M</td>
<td>IAD2-RD</td>
</tr>
<tr>
<td>24.</td>
<td>320</td>
<td>T</td>
<td>A</td>
<td>IAD2-RD</td>
</tr>
<tr>
<td>25.</td>
<td>321</td>
<td>P</td>
<td>S</td>
<td>IAD2-RD</td>
</tr>
</tbody>
</table>

Single letter codes for amino acids and significant changes in bold are mentioned. Accession no. of IRF-2: mouse (J03168), human (NM_002199) and rat (AB003091). DBD, DNA-binding domain; TAD, transcriptional activation domain; RD, repression domain; IAD2, IRF association domain.

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Figure 1. (A) Changes in amino acids in IRF-2 of mouse (M), human (H) and rat (R). The vertical bars represent IRF-2 and the horizontal lines inside the bar represent amino acid changes. The stars represent absence of amino acid at that position. Mouse IRF-2 is taken as the reference for comparison with human and rat IRF-2. Changes in murine IRF-2 with respect to human and rat IRF-2 are also mentioned. Rat IRF-2 sequence is partial. (B) Uninduced (−) and IPTG-induced (+) cell extracts from vector, wt IRF-2 (95, 99, 108) and mt IRF-2 (95M7, 95M9) clones, were analysed by 10% SDS-PAGE along with molecular size marker (lane 3). (C) Expression of GST, wt, mt GST-IRF-2 and the proteolytic degradation products are indicated. Uninduced (−) and IPTG-induced (+) cell extracts (2.5 and 5.0 μg) from vector, wt IRF-2 (95) and mt IRF-2 (95M7) clones were separated by 10% SDS-PAGE, western-blotted, probed with anti-GST or anti-IRF-2 (D) antibody followed by anti-IgG-HRP antibody and developed by DAB-staining.
There are total 25 amino acid variations, 2 in DBD, 5 in DBD/TAD, 2 in TAD, 3 in IAD and 13 in IAD/RD. This indicated that during evolution, IRF-2 had diversified in terms of binding to other IRFs and transcriptional repression, both of these two aspects involve protein–protein interactions. Several two consecutive amino acid variations are observed in mouse IRF-2 vs. human IRF-2, i.e. at positions 131-132 (ER vs. DK), 153-154 (GF vs. DL), variations are observed in mouse IRF-2 vs. human IRF-2, protein interactions. Several two consecutive amino acid in terms of binding to other IRFs and transcriptional

314SSSM317, which were used for immunoblotting and DNA binding. We hypothesized that the tetrapeptide sequence at 314-317 may be important for IRF-2 function. In order to test this, we expressed recombinant GST-IRF-2 with 314PAPV317 and may be important for IRF-2 function. In order to test this, expressed recombinant GST-IRF-2 with 314PAPV317 and 314SSSM317, which were used for immunoblotting and DNA binding.

3.2 Expression of wt and mt GST-IRF-2

Figure 1B shows the SDS-PAGE expression profile of wt GST-IRF-2 from the 95, 99 and 108 clones (lanes 2, 7, 9) and mt GST-IRF-2 from the 95M and 99M clones (lanes 11, 13) as ~ 66 kDa fusion proteins in comparison to GST (26 kDa) from the vector (V, lane 5) after IPTG-induction (+). Additional IPTG-inducible bands are observed, for example, at 62, 32, 30 kDa, which represent proteolytic degradation of GST-IRF-2. The size and expression level of wt IRF-2 and mt IRF-2 proteins are comparable. Comparing to our earlier report (Upreti and Rath 2005) of expression of GST-IRF-1, we observed that GST-IRF-2 showed less proteolytic degradation possibly because of variations in the C-terminal regions of the two transcription factors.

3.3 Immunoreactivity of wt and mt GST-IRF-2

Figure 1C and 1D show immunoreactivity of wt and mt GST-IRF-2 with anti-GST and anti-IRF-2 antibodies by western blotting. Anti-GST immunoblot shows that 5 and 2.5 μg of IPTG-induced cell extracts from mt IRF-2 (95M) and wt IRF-2 (95) clones contained intact GST-IRF-2 fusion proteins as a single higher band and their degradation products as the lower bands in a comparable manner (figure 1C). The anti-IRF-2 immunoblot shows that both the mt (95M) and wt (95) GST-IRF-2 proteins were again detected as a single intact strong band with some weakly detected lower bands as degradation products again in a comparable manner (figure 1D). The vector clone and the uninduced wt and mt clones do not show any immunoreactivity. The anti-GST antibody detected the N-terminal part while the anti-IRF-2 antibody detected the C-terminal part of the wt and mt GST-IRF-2 fusion proteins. Hence, there was no difference in immunoreactivity of wt and mt IRF-2 proteins.

3.4 DNA-binding activity of wt and mt GST-IRF-2

DNA-binding activity of IPTG-induced cell extracts from wt and mt GST-IRF-2 with [32P]-labelled (GAAAGT)4 was measured by EMSA and the DNA–protein complexes were quantified for comparison. Figure 2A shows three independent EMSA experiments (a, b, c), with three wt (95, 99, 108) and two mt (95M7, 95M9) GST-IRF-2 clones under identical conditions. The wt IRF-2 show five (1-5) DNA–protein complexes (lanes 5, 8, 11) and mt IRF-2 show 5 DNA–protein complexes of higher molecular mass (lanes 14, 17); in both, the complexes 3 and 4 are strongly detected. The vector (lanes 2, 3) and uninduced extracts (lanes 4, 7, 10) do not show any complex. There is a little leaky expression of mt IRF-2 (lanes 13, 16) in the absence of IPTG. The complexes are competed by 100-fold molar excess of (GAAAGT)4 completely in wt IRF-2 (lanes 6, 9, 12) but incompletely (lane 15) or completely (lane 18) in mt IRF-2. Figure 2B shows quantitation of the DNA–protein complexes (1-5) shown in figure 2A as %pixel intensity of free oligo. Interestingly, complexes 3 and 4, the two major strong complexes observed in figure 2A, are formed in greater quantities by mt IRF-2 (30–35%) than by wt IRF-2 (20%), whereas the complexes 1, 2 and 5 are formed in comparable quantities. Hence, mt IRF-2 can form more and higher DNA–protein complexes with (GAAAGT)4.

3.5 Competition of wt and mt IRF-2-DNA complexes by (GAAAGT)4

Figure 3A and 3C show kinetics of competition of IRF-2-DNA complexes by IRF-E sequence by EMSA and Figure 3B and 3D show quantitation of the complexes as % pixel of free oligo. The wt and mt GST-IRF-2 complexes were formed with [32P]-labelled (GAAAGT)4 for 30 min and competed by 100-fold molar excess of (GAAAGT)4 for 5–30 min at 37°C. Figure 3A shows that wt IRF-2 complexes (1-5) are formed by 30 min at 37°C (lane 2); the complexes are stable if the mixture is further incubated on ice (0°C) for a subsequent 30 min (lane 3) but dissociate on further incubation at 37°C for 30 min (lane 4). Within 5 min of further incubation at 37°C, the complexes are competed by 100-fold (GAAAGT)4 (lane 5), compare 30 min with 30+5 min in B. The competition is maximal by 15 min (lane 7), leaving only a trace amount of complex 3 up to 30 min (lanes 8-10), compare 60 min with 30+30 min in B. Figure 3C shows that mt GST-IRF-2 complex 2 increased in amount from the initial 30 min binding at 37°C to further incubation for 30 min on ice, and this effect was not observed in wt GST-IRF-2, compare 30 min to 30+30* min in D vs B. The mt GST-IRF-2 complexes 3 and 4 still remain up to 5–15 min (lanes 5-7) after competition by 100-fold (GAAAGT)4 and decrease by 30 min (lane 10), compare
Figure 2. (A) DNA-binding activity of wt- and mt IRF-2 by EMSA. IPTG-induced (+) cell extracts (2.0 μg) from wt (95, 99, 108) and mt (95M7, 95M9) GST-IRF-2 clones were bound with [32P](GAAAGT)₄ and the DNA–protein complexes were resolved by 7.5% native PAGE. Unlabelled oligo (100-fold) were used for competition. Three independent experiments are shown in (a), (b) and (c). GST-IRF-2 complexes 1-5 are indicated. (B) Quantitation of wt and mt GST-IRF-2-DNA complexes from the gels in (A) by phosphoimager as %pixel intensity of free oligo was expressed in mean value ± SD.
Figure 3. Kinetics of competition of wt (A) and mt (C) IRF-2-DNA complexes by unlabelled oligo. IPTG-induced (+) cell extracts (2 μg) from wt (95) and mt (95M7) GST-IRF-2 clones were bound with [32P]-C-terminal tetrapeptide infl uences DNA binding of IRF-2
GAAAGT)₄(100X) at 37°C for 30 min (lane 2), further incubated for 30 min (30*) on ice (lane 3) or 37°C (lane 4), competed with 100-fold molar excess of (GAAAGT)₄ for 5-30 min at 37°C (lane 5-10), respectively. Lane 1 shows free oligo. Complexes 1-5 are indicated. (B) and (D) show quantitation of wt and mt IRF-2-DNA complexes from the gels in (A) and (C) as %pixel intensity of free oligo.

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30 min with 30+5 min to 30+15 min in D. Also, the decrease in complexes from 30 to 60 min is more in wt IRF-2 than in mt IRF-2, indicating slower dissociation of mt IRF-2 than wt IRF-2. Hence mt IRF-2 can bind the (GAAAGT)$_4$ sequence more strongly than wt IRF-2.

3.6 Resolution of wt and mt IRF-2-DNA complexes by anti-IRF-2 antibody

Because wt GST-IRF-2 and mt GST-IRF-2 are expressed as same-size proteins in SDS-PAGE but they formed different IRF-2-DNA complexes, we hypothesized that the difference in the DNA–protein complexes may be due to changes in conformational properties of the proteins. We, therefore, checked DNA–protein complex formation in the presence of anti-IRF-2 antibody raised against the C-terminal region. Figure 4 shows complex formation by wt IRF-2 (A) and mt IRF-2 (B) in the presence of 0.2 μg of anti-IRF-2 antibody. Interestingly, wt and mt GST-IRF-2 formed two strong complexes (named as complex 2* and 3*) (lane 4) in the presence of anti-IRF-2 antibody, the 2* and 3* complexes migrated as complexes 3 and 5 for wt IRF-2, and in appeared between complexes 3 and 4 and cut the same position as complex 5 for mt IRF-2, respectively. The complex 1* most likely represents the supershifted (SS) complex for wt and mt IRF-2. This indicates that the difference in mobility between wt and mt IRF-2-DNA complexes is due to conformational alterations in the IRF-2, which is resolved by anti-IRF-2 antibody. Therefore, $^{314}$PAPV$^{317}$ and $^{314}$SSSM$^{317}$ sequences in the background of same IRF-2 sequence can form different DNA–protein complexes of altered conformations. Thus, alterations in the C-terminal region of IRF-2 influenced its DNA-binding activity of the N-terminal region.

3.7 Role of N-terminal and C-terminal regions in DNA-binding activity of IRF-2

The crystal structure of IRF-2-DBD and DNA complex at 2.2 Å resolution (Fujii et al. 1999) showed that DBD...
of IRF-2 consists of four-stranded anti-parallel β sheets (β1–β4) with three α helices (α1–α3) and three long loops (L1-L3), and resembles a winged helix-turn- helix (HTH) motif. All these six domains interacted with the DNA. The His-40 of L1 interacted with GAAA at the minor groove, and the 5′-fl anking AA located 2 bp upstream of the GAAA core sequence was recognized by the α3-helix. The AA was part of another upstream core sequence recognized by another DBD at the major groove. As His-40 is conserved in the IRF family, a similar recognition was proposed to occur in all IRFs and the AANNGAAA sequence was proposed to be consensus IRF-element (IRF-E) recognized by IRF-DBDs. The contacts with the major groove of the GAAA sequence were localized at the C-terminal half of the recognition helix and were mediated by four residues: Asn-80, Arg-82, Cys-83 and Asn-86. An extensive network of water molecules existed within the interface between the recognition helix and the DNA. The second adenine (GAAA) was recognized by the conserved Cys-83 and Asn-86 through hydrogen bonding with water molecule. The DBD of IRF-1 and IRF-2 have five conserved tryptophans (W), which also take part in DNA binding.

Our study shows that by replacing \(314\text{PAPV}^{317}\) by \(314\text{SSSM}^{317}\) in the C-terminal region, the N-terminal DNA-binding activity of GST-IRF-2 is altered possibly because of conformational change brought about by intramolecular interactions between the C-terminal and the N-terminal regions of IRF-2. This alteration is resolved by anti-IRF-2 antibody used in the study. The PAPV sequence of mouse IRF-2 is more nonpolar and hydrophobic, whereas the SSSM sequence of human IRF-2 is more polar and hydrophilic. The PAPV sequence may also provide less flexibility owing to the two prolines. Therefore, alterations in the charge, hydrophobic/hydrophilic property and the flexibility of these two tetrapeptide sequences may contribute to alterations in the interaction between the C-terminal region and the N-terminal region of IRF-2, which may influence its DNA-binding activity. In addition, the three Serine residues in human IRF-2 may also provide sites for phosphorylation by Serine/Threonine kinases. Also, the methionine may provide a unique mark in human IRF-2. This SSSM is not available in mouse IRF-2. Earlier we reported a unique C-terminal hexapeptide sequence, \(\beta^{281}\text{MQMDII}^{293}\) in mouse IRF-1, which is different from \(\beta^{197}\text{IPVEVV}^{202}\) in human IRF-1, and this difference resulted in differential effects on the expression of IFN-β, iNOS and Cox-2 genes (Upreti et al. 2004). Thus, activities of IRF-1 and IRF-2 are regulated by small domains/regions within the proteins (Palombella and Maniatis 1992; Naguyen et al. 1995; Xi and Blanck 2003; Eckert et al. 2006) and variations in short peptide motifs in highly homologous transcription factors during evolution may alter their activity in two different species.

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