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IRF-1 and IRF-4 Regulate Target Gene Expression via Interaction with DMP1

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Interferon regulatory factor-4 (IRF-4), a member of the IRF gene family, is a lymphoid-specific transcription factor. IRF-4 deficient mice showed severe immunodeficiencies. Both B- and T-cell activation were profoundly affected: serum immunoglobulin concentrations and antibody responses were decreased, and cytotoxic and antitumor responses were absent in IRF-4 knockout mice. Thus, IRF-4 is essential for the functions of mature B- and T-lymphocytes.

To analyze IRF-4 function, we searched for IRF-4-interacting factors. A plasmid was constructed to express a LexA-IRF-4 fusion protein from the inducible *GAL1* promoter in yeast cells. When this low copy plasmid *(pGilda/IRF-4)* was introduced into the yeast strain NOY397, cell growth was significantly inhibited. We supposed that this growth inhibition arose from interference with cellular factors essential for cell viability. We isolated several genes that could rescue the growth inhibition phenotype by screening a yeast genomic library. One of the clones, encoding the REB1 protein, interacted with IRF-4 *in vitro*. The yeast REB1 protein shares homology with the human transcription factor DMP1. IRF-4 was shown to interact *in vitro* with DMP1, via its DNA binding domain. IRF-1 also interacted with DMP1. These results suggest that IRF-1 and IRF-4 might regulate target gene expression, via interactions with DMP1.

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Introduction

Regulation of gene expression can occur at several different levels, but the activation of gene-specific transcription factors is considered to be fundamentally essential to this process. One family of transcription factors, the interferon regulatory factors (IRFs), consists of ten members. All of the members of this family share homology in their first 115 amino acids, encompassing the DNA binding domain, which contains a characteristic repeat of five tryptophan residues spaced by 10-18aa[1－3)]. IRFs modulate the interferon (IFN) response by binding to *IRF* response elements within the promoters of *IFN* genes and interferon-stimulated genes, which regulate the host response to pathogens, cellular proliferation, cytokine signaling, and apoptosis. The gene encoding IRF-4 was cloned as a lymphoid-specific IRF (LSIRF)[4)], expressed at all stages of B-cell development and in mature T cells. Like many other IRF-/- mice, IRF-4-deficient mice exhibited severe immunodeficiencies. A normal T- and B-cell distribution was observed at 4 to 5 weeks of age, but with time, IRF-4-/- mice gradually exhibited severe lymphadenopathy. Both B- and T-cell activation were profoundly affected: serum immunoglobulin concentrations and antibody responses were decreased, and cytotoxic and antitumor responses were absent in IRF-4 knockout mice[5)]. Thus IRF-4 appears to be essential for the function and homeostasis of both mature B- and T- lymphocytes. IRF-4 and the hematopoietic-specific Ets protein, PU.1, form complexes in B cells on composite elements present in immunoglobulin light chain *(IgL)* gene enhancers[6,7)]. Recently, it was reported that macrophages express IRF-4, and together with PU.1, these factors synergistically activated an *IL-1β* reporter gene[8－10)]. IRF-4 interacts with E47, a component of the E2A transcription factor. In a reporter gene assay, IRF-4 and E47 bound together to the immunoglobulin *κ 3′* enhancer region, and they synergistically generated a 100-fold increase in

transcriptional activity[11].

DMP1 is an unusual, poorly characterized transcription factor that can induce ARF-dependent cell cycle arrest. DMP1 was isolated in a two-hybrid interactive screen using cyclin D2 as bait, and its gene encodes a 120-130 kD nuclear phosphoprotein, with a central DNA-binding domain containing three Myb-like repeats flanked by acidic transactivation domains at both termini[12~15]. The human and murine orthologs share 95% amino-acid sequence identity, and are completely conserved throughout their Myb-like repeats[16]. The DMP1 protein binds to nonameric Ets consensus sequences in DNA *(CCCG[G or T]ATGT)*, and competes with Ets-family proteins for sites that contain the *GGA* core. DNA binding by DMP1 can be antagonized by the interactions with D-type cyclins, but not with other cyclins. DMP1 expression induces the ARF tumor suppressor gene in mouse fibroblasts, leading to cell cycle arrest in a p53-dependent manner. Although DMP1-null mice did not spontaneously develop cancers in their first year of life, they were susceptible to carcinogen- and radiation- induced tumors, implying that by regulating ARF function, DMP1 may have tumor- suppressing activities[17]. Here we show that DMP1 binds to IRF *in vitro*, and together with IRF, DMP1 synergistically activates the IRF target gene promoter.

Materials and Methods

Yeast strains and plasmids

Assays were carried out with the yeast strains NOY397 *(MATa, MATa, ade2-1, ura3-1, his3-11, trp1-1, leu2-3 112, can1-100)* and Y153 *(MATa, leu2-3 112, ura3-52, trp1-901, his3-delta200, ada2-101, gal4delta, gal80delta, URA3::GAL1-lacZ, LYS2::GAL-HIS3)*. The plasmid expressing GAL4DB-IRF-4 in *S. cerevisiae* was constructed by ligating the full-length *IRF-4* cDNA into the *EcoRI* site of *pAS2-1* (2μ origin; *ADH1* promoter, CLONTECH Laboratories, USA). The plasmid expressing LexA-IRF-4 in *S. cerevisiae* was constructed by ligating the full-length *IRF-4* cDNA into the BamHI site of pGilda (*CEN/ARS* origin; *GAL1* promoter, CLONTECH Laboratories, USA). Transformations were performed by the lithium acetate method. The LexA-IRF-4 fusion protein was induced in the absence of glucose and the presence of galactose.

Complementation of yeast-growth inhibition

The LexA-IRF-4 transformant strain was transformed with a yeast genomic library on the 2μm vector *Yep13* (donated by Y. Nogi), and 40 faster-growing colonies were isolated on minimal medium with 2% galactose. Among them, four clones contained the region between 339,100 bp to 349,101 bp on chromosome II. We constructed several subclones from this region. An *Nhe*I fragment and an *Nhe*I-*Apa*I fragment from this clone, when cloned into Yep13, could not rescue the yeast-growth inhibition by IRF-4. However, an *Xho*I fragment from this clone could rescue yeast-growth inhibition by IRF-4. This *Xho*I fragment contained the entire *REB1* gene.

Plasmid constructs

pcDNA3/REB1 was constructed by PCR amplification for *in vitro* transcription. *pGEX/IRF-4* was constructed by ligating the full-length *IRF-4* gene into *Bam*HI-*Eco*RI cleaved *pGEX4T3*. The GST-IRF-4 deletion construct, *pGEX/IRF-4(1-125)*, was constructed by ligating a *Bam*HI-*Bsr*GI fragment, cleaved from *pGEX/IRF-4*, into *pGEX4T3*. *pGEX/IRF-4(1-277)* was constructed by ligating a *Bam*HI-*Nco*I fragment, cleaved from *pGEX/IRF-4*, into *pGEX4T3*. *pGEX/IRF-4(277-450)* was constructed by ligating an *Nco*I-*Eco*RI fragment from *pGEX/IRF-4* into *pGEX4T3*. *pGEX/IRF-4(125-450)* was constructed by ligating a *Bsr*GI-*Eco*RI fragment from *pGEX/IRF-4* into *pGEX4T3*. *pGEX/IRF-4(125-277)* was constructed by ligating a *Bsr*GI-*Nco*I fragment from cleaved *pGEX/IRF-4* into *pGEX4T3*. *pGEX/IRF-4(1-125,277-450)* was constructed by self-ligation after *Bsr*GI-*Nco*I cleavage of *pGEX/IRF-4*. To produce the of GST fusion proteins, *DMP1* and *IRF-1* were amplified by PCR and subcloned into *pGEX-4T2*. To construct the expression plasmids, *DMP1* and *IRF-1* were amplified by PCR and subcloned into *pcDNA3*. The *GST-DMP1* deletion construct, *pGEX/DMP1(1-172)*, was constructed by ligating a *Bam*HI-*Afl*II fragment from *pGEX/DMP1* into *pGEX-4T2*. *pGEX/DMP1(1-313)* was constructed by ligating a *Bam*HI-*Kpn*I fragment from *pGEX/DMP1* into *pGEX-4T2*. *pGEX/DMP1(173-458)* was constructed by ligating an *Afl*II-*Nco*I fragment from *pGEX/DMP1* into *pGEX-4T2*. *pGEX/DMP1(173-756)* was constructed by ligating a fragment from *Bam*HI-*Afl*II cleaved *pGEX/DMP1* into *pGEX-4T2*. *pGEX/DMP1(458-756)* was constructed by ligating an *Nco*I-*Bam*HI fragment from *pGEX/DMP1* into *pGEX-4T2*. The 2'-5'*OAS-Luci* and *GBP(-216)-Luci* reporter plasmids were both donated by Dr. T.Yamagata[26]. The PU.1 expression plasmid *(pCMV5/PU.1)* and the *pIL-1 β-Luci* reporter plasmid were both donated by Dr. M.J.Fenton[9, 10].

Protein expression

Protein synthesis by *in vitro* transcription and translation was carried out with the TNT-coupled reticulocyte lysate system (Promega, WI), according to the manufacturer's recommendations. Newly synthesized [^{35}S]labeled proteins were analyzed by SDS-PAGE, followed by visualization by phosphoimager analysis (Fuji BAS2000 phosphoimager).

In vitro protein-protein interaction assay

Escherichia coli BL21 cells harboring the various GST fusion constructs were grown to an OD_{600} of 0.5 at 30℃, and were induced with 2 mM isopropyl β-D-thiogalactopyranoside (IPTG) for an additional 2h. Cells were harvested in PBS and lysed by sonication, and the lysate was cleared by centrifugation at 8,000 ×g for 10min. Cell lysates containing fusion proteins were incubated with 300 μl of 50% glutathione-Sepharose beads (Pharmacia Biotech, Piscataway, NJ), in a final volume of 1000 μl, for 1 h at 4℃ with gentle rotation. The beads were then washed three times with DBT buffer (20 mM Hepes-KOH (pH 7.9), 100 mM KCl, 0.5 mM EDTA, 20% glycerol, 0.2% Triton X-100, 0.5 mM PMSF and 1 mM DTT), and subsequently, a 60 μl aliquot of IVT [^{35}S]methionine-labeled target protein was added for an additional 3h. The beads were washed three times with 200 μl of DBT-0.3 buffer (20 mM Hepes-KOH (pH 7.9), 300 mM KCl, 0.5 mM EDTA, 20% glycerol, 0.2% Triton X-100, 0.5 mM PMSF and 1 mM DTT), and the bound proteins were eluted and separated by 12% SDS-PAGE, followed by visualization by phosphoimager analysis.

Cell lines, transfections, and luciferase assays

For transfections, all plasmids were prepared by the alkaline-SDS method, followed by purification with Qiagen columns (Qiagen, Valencia, CA). HeLa and N-Tera2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For luciferase reporter assays, HeLa or N-Tera2 cells were seeded at a density of 2 ×10^5 per 6-cm plate. Cells were transfected 18 hours after seeding with the effector plasmids, along with the reporter plasmids and *pRL-β actin*, an expression vector of renilla luciferase, using the FUGENE™6 Transfection Reagent (Roche Molecular Biochemicals, Tokyo, Japan). The total amounts of DNA for each transfection were equalized by the addition of an empty vector. Luciferase assays were performed by using the Dual-Luciferase Reporter System (Promega, Madison, WI), in which the relative luciferase activities were calculated by normalizing the transfection efficiency according to the renilla luciferase activities. All transfection experiments were performed at least 3 times, and similar results were obtained.

Results

IRF-4 severely inhibits yeast cell growth

To characterize the IRF-4 function, we searched for IRF-4-interacting factors. A plasmid was constructed to express GAL4-IRF-4 from the constitutive *ADH1* promoter *in vivo*. When this high copy plasmid *(pAS2-1/IRF-4)* was introduced into the yeast strain Y153, cell growth was strongly inhibited (Fig.1A). A plasmid was constructed to express the LexA-IRF-4 fusion protein from the inducible *GAL1* promoter in yeast cells.

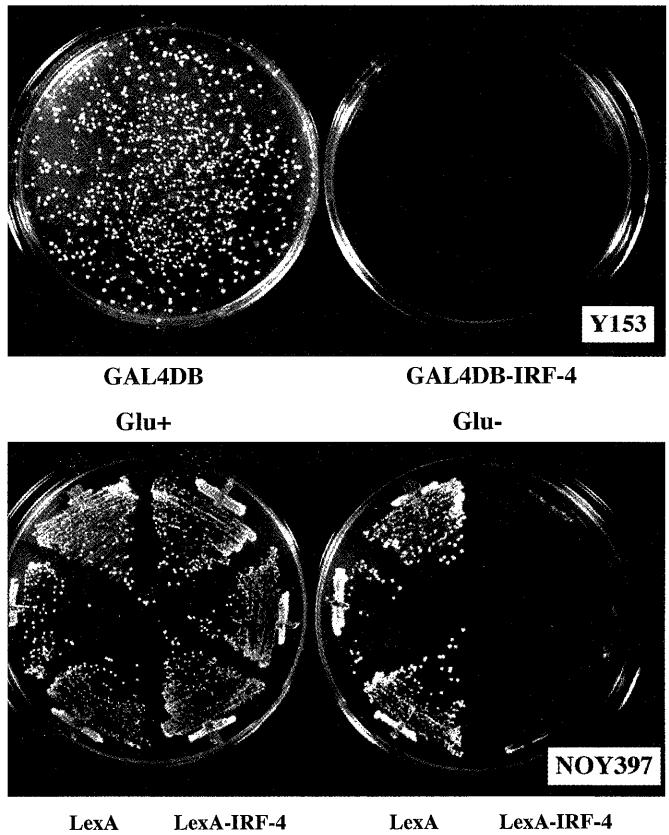

Figure 1. IRF-4 severely inhibits yeast cell growth. A plasmid was constructed to express GAL4-IRF-4 from the constitutive *ADH1* promoter *in vivo*. When this high copy plasmid *(pAS2-1/IRF-4)* was introduced into the yeast strain Y153, cell growth was strongly inhibited (Fig.1A). A plasmid was constructed to express a LexA-IRF-4 fusion protein from the inducible *GAL1* promoter in yeast cells. When this low copy plasmid *(pGilda/IRF-4)* was introduced into the yeast strain NOY397, cell growth was severely inhibited (Fig. 1B). The LexA-IRF-4 fusion protein was induced in the absence of glucose and the presence of galactose.

When this low copy plasmid *(pGilda/IRF-4)* was introduced into the yeast strain NOY397, cell growth was severely inhibited (Fig. 1B). We concluded that this growth inhibition occurred from the interference with cellular factors essential for cell viability.

The yeast REB1 protein rescues the IRF-4 mediated growth inhibition

We supposed that the yeast growth inhibition with IRF-4 was a consequence of interference with cellular factors essential for cell viability. To confirm our hypothesis, the LexA-IRF-4 transformant strain was transformed with a yeast genomic library on a Yep13 vector. We screened approximately 5×10^5 yeast transformants. Among 40 faster-growing colonies that were isolated, four clones contained the region between 339,100 bp to 349,101 bp on chromosome II. We constructed several subclones from this clone. The only subclone that could rescue the growth inhibition phenotype by IRF-4 contained the *REB1* gene (data not shown).

The REB1 protein interacts with IRF-4 in vitro

In vitro glutathione S-transferase (GST) pull-down experiments were carried out using full-length REB1 and a GST-IRF-4 fusion protein (Fig. 2). The REB1 protein interacted with IRF-4. In order to map the REB1-interaction domain on IRF-4, a series of deletion proteins was tested for their ability to interact with REB1. The N-terminal DNA binding domain contained in GST-IRF-4 1-277 was sufficient to mediate this interaction.

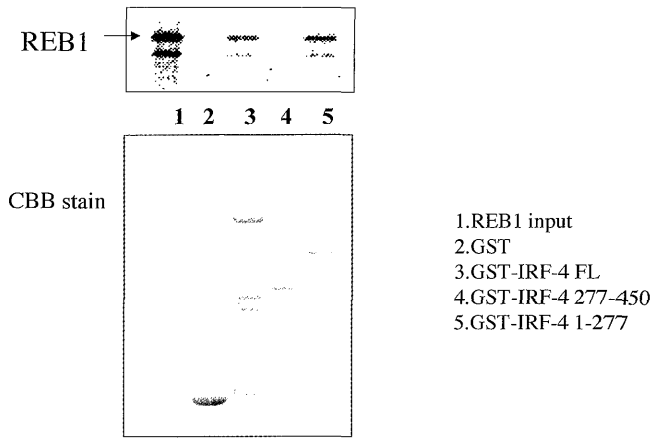

Figure 2. The REB1 protein interacts with IRF-4. The REB1 protein was prepared by *in vitro* transcription and translation in the presence of [^{35}S]-labeled methionine. GST pull-down analyses of full-length REB1 with GST and the GST-IRF-4 fusion proteins. Eluted proteins were subjected to SDS-PAGE. Lane 1 shows aliquots of the input radioactive proteins, corresponding to 10% of the amount actually used in each of the subsequent binding reactions.

DMP1 interacts with IRF-4 in vitro

The Yeast REB1 protein shares homology with the human transcription factor DMP1 (Table 1). Therefore, we sought to determine whether DMP1 interacts with

Table 1. Protein sequence comparisons between REB1 and hDMP1

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 Identities = 27/106(25%), positives = 57/106 (53%)

REB1  691   INWTIVSERMGGTRSRIQCRYKWNKLVKREAIAKIQTVKDDDMLWIFEKLRDLGITEDSQ
            ++    +  +                +  +      +    + ++      ++ +  + +++
hDMP1 304   VSWAAVAERVG-TRSEKQCRSKWLNYLNWKQSGGTEWTKEDEINLIL-RIAELDVADEND

REB1  751   VDWDELAALKPGMKLNGLELKLCYERMKKKVKGYKQKSINEISKEL
            ++           ++ +        +  + +++  +        +
hDMP1 362   INWDLLAEGWSSVR-SPQWLRSKWWTIKRQIANHKDVSFPVLIKGL


 Identities = 31/89(34%), positives = 44/89 (49%)

REB1  437   SSDRPKDNFWNNIYKVLPYRSSSSIYKHMRRKYHIFEQRGKWTAEEEQELAKLCAEKEGQ
                  +              ++ + +             +      ++       +
hDMP1 188   SKDERKD-FYRTIAWGLN-RPLFAVYRRVLRMYDDRNHVGKYTPEEIEKLKELRIKHGND

REB1  497   WAEIGKTLGRMPEDCRDRWRNYVK--CGTNR
                        +       +       +
hDMP1 246   WATIGAALGRSASSVKDRCR-LMKDTCNTGK
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(+ denotes conservative amino acid substitution)

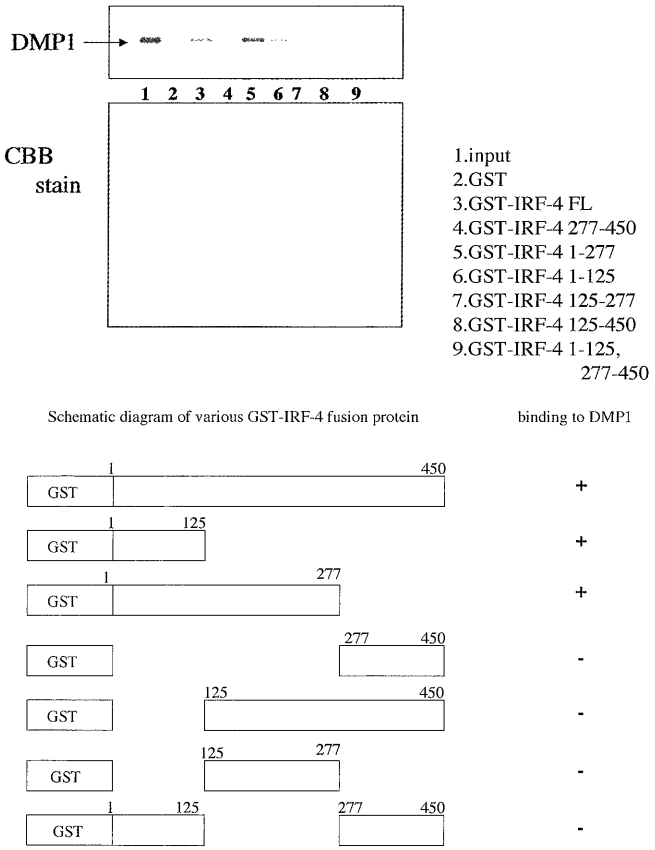

Figure 3. The DMP1 protein can physically interacts with IRF-4. (A) Schematic illustration of the domain structures of the fusion proteins. (B) The DMP1 protein was prepared by *in vitro* transcription and translation in the presence of [^{35}S]-labeled methionine. GST pull-down analyses of full-length REB1 with GST and the GST-IRF-4 fusion proteins. Eluted proteins were subjected to SDS-PAGE. Lane 1 shows aliquots of the input radioactive proteins, corresponding to 10% of the amount actually used in each of the subsequent binding reactions.

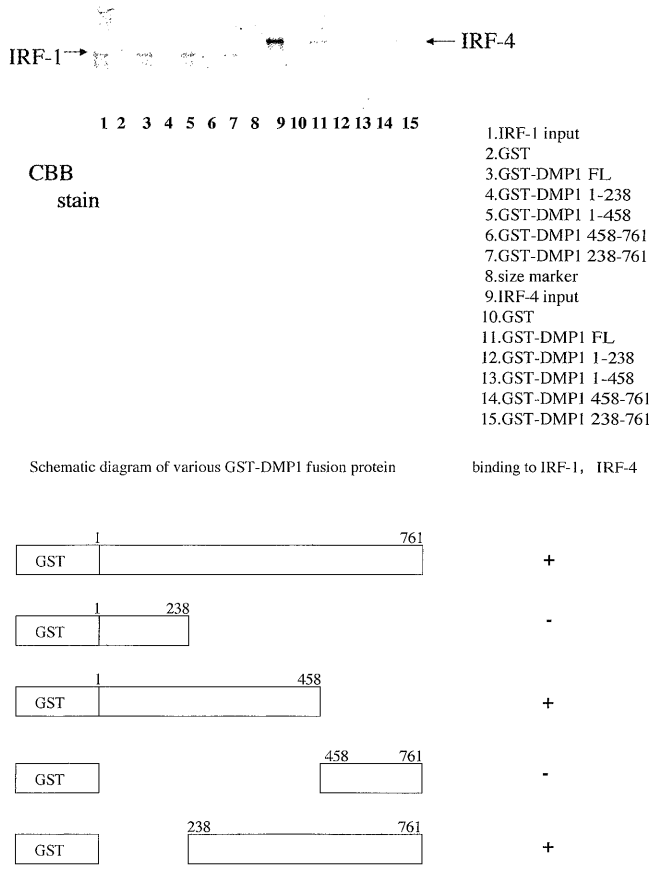

Figure 4. DMP1 interacts with IRF-1 and IRF-4 through the DNA binding domain. (A) Schematic illustration of the domain structures of the fusion proteins. (B) The IRF-1 and IRF-4 proteins were prepared by *in vitro* transcription and translation in the presence of [^{35}S]-labeled methionine. GST pull-down analyses of full-length IRF-1 and IRF-4 with GST and GST-DMP1 fusion proteins. Eluted proteins were subjected to SDS-PAGE. Lane 1 shows aliquots of the input radioactive proteins, corresponding to 10% of the amounts actually used in each of the subsequent binding reactions.

IRF-4. The full-length IRF-4 and its deletion proteins were fused to GST, and we analyzed the ability of these fusion peptides, bound to glutathione-Sepharose beads, to retain ^{35}S-labeled DMP1 prepared by IVT (Fig. 3). The DMP1 protein interacted with IRF-4, and the N-terminal DNA binding domain contained in GST-IRF-4 1-125 was sufficient to mediate this interaction. In order to map the IRF-4-interaction domain on DMP1, a series of DMP-1 deletion proteins was tested for their ability to interact with IRF-4 (Figure. 4). The DNA binding domain contained in GST-DMP1 238-458 was sufficient to mediate this interaction.

DMP1 interacts with IRF-1 in vitro

IRF-4 shares homology with IRF-1, another IRF family transcription factor, and therefore we wished to determine whether DMP1 also interacts with IRF-1. *In vitro* GST pull-down experiments, carried out using full-length IRF-1 and a GST-DMP1 fusion protein, revealed that DMP1 also interacts with IRF-1 (Fig. 4). In order to map the IRF-1-interaction domain on DMP1, a series of deletion proteins was tested for their ability to interact with IRF-1. The DNA binding domain contained in GST-DMP1 238-458 was sufficient to mediate this interaction.

DMP1 enhances IRF transcriptional activity

Since DMP1 has the potential to bind to both IRF-1 and IRF-4, we investigated its effect on the various promoters of IRF target genes. A number of cotransfection experiments were performed with N-

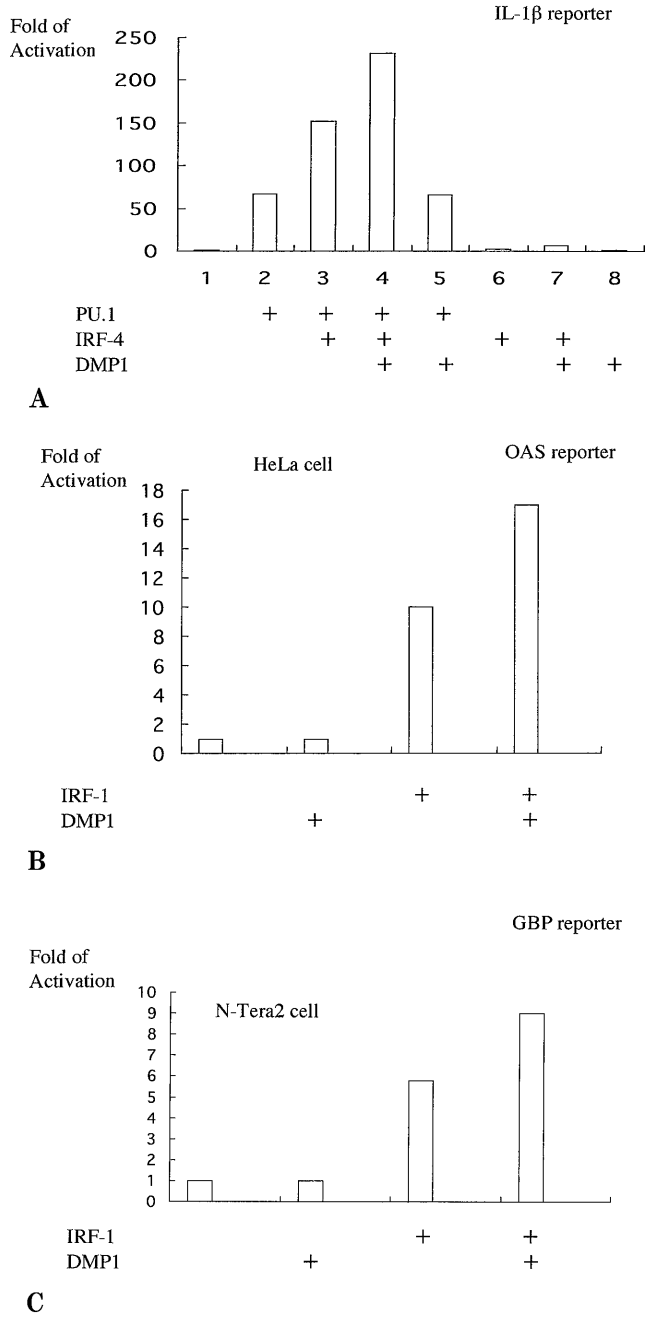

Figure 5. DMP1 enhances the transcriptional activities of IRF-1 and IRF-4. (A) HeLa cells were cotransfected with the *IL-1β* promoter-Luci reporter plasmid, with an internal control luciferase vector, together with various combinations of the *PU.1*, *IRF-4*, and DMP1 plasmids. Cells were harvested 48 hours after transfection. (B) HeLa cells were cotransfected with the *2'-5'OAS-Luci* reporter plasmid, with the internal control plasmid, together with various combinations of the *IRF-1* and *DMP1* plasmids. (C) N-Tera2 cells were cotransfected with the *GBP-Luci* reporter plasmid, with the internal control plasmid, together with various combinations of the *IRF-1* and *DMP1* plasmids. All transfection experiments were performed at least 3 times, and representative results are shown.

Tera2 cells and HeLa cells. The transcription factor PU.1 and IRF-4 synergize to mediate transcriptional activation of the human *IL-1β* gene. As shown in Fig.5, coexpression of PU.1, IRF-4, and DMP1 synergized to induce a high level of *IL-1β* promoter activity in HeLa cells. We cotransfected HeLa cells with the 2'-5'*OAS-Luci* reporter plasmid and the *IRF-1* and *DMP1* expression plasmids. As shown in Fig.5, overexpression of IRF-1 enhanced the 2'-5'*OAS(-159)* promoter activity up to 10-fold over the basal activity. When DMP1 was coexpressed, the promoter activity was stimulated up to 20-fold over the basal activity. We cotransfected N-Tera2 cells with the *GBP-Luci* reporter plasmid and the *IRF-1* and *DMP1* expression plasmids. IRF-1 activated the *GBP(-216)* promoter up to 6-fold over the basal activity in the N-Tera2 cells (Fig. 5). When DMP1 was coexpressed, the promoter activity was stimulated up to 10-fold over the basal activity.

Discussion

To clarify the function of IRF-4, we searched for IRF-4-interacting factors. A plasmid was constructed to express a LexA-IRF-4 fusion protein from the inducible *GAL1* promoter in yeast cells. When this low copy plasmid *(pGilda/IRF-4)* was introduced into the yeast strain NOY397, cell growth was severely inhibited. Our results are similar to those obtained by Berger *et al.*[18], who reported that the expression of high levels of GAL4-VP16 strongly inhibited the growth of yeast cells. They showed that this inhibition resulted from the trapping of essential, general transcription factors at genomic sites to which GAL4 would bind. We supposed that the yeast growth inhibition by IRF-4 was a consequence of interference with cellular factors essential for cell viability. To confirm our hypothesis, we screened a yeast genomic library, using the methods for complementation of yeast-growth inhibition. This method is a newly developed genetic strategy.

We isolated the *REB1* gene, which could rescue the IRF-4-mediated growth inhibition phenotype. REB1 not only rescued the growth inhibition by IRF-4, but also *in vitro*-translated REB1 bound directly to the GST-IRF-4 fusion protein. The *Saccharomyces cerevisiae* REB1 is a DNA binding protein that has been implicated in the activation of transcription by RNA polymerase II, the termination of transcription by RNA polymerase I, and the organization of nucleosomes. REB1 is an essential protein for yeast cell growth, and contains an unusual DNA binding region, consisting

of two Myb-like domains separated by nearly 150 amino acids[19, 20].

The yeast REB1 protein shares homology with the human transcription factor DMP1. DMP1 was isolated in a two-hybrid interactive screen using cyclin D2 as bait, and it encodes a 120-130 kD nuclear phosphoprotein, with a central DNA-binding domain containing three Myb-like repeats flanked by acidic transactivation domains at both termini.

We have demonstrated that the DMP1 interacts with IRF-4. From a deletion mutant analysis, we found that the N-terminal DNA binding domain (1-125) of IRF-4 and the DNA binding domain (238-458) of DMP1 were sufficient to mediate this interaction. Since IRF-4 shares homology with IRF-1, another IRF family transcription factor, we postulated that DMP1 also interacts with IRF-1. Using GST pull-down experiments, we confirmed that DMP1 indeed interacts with IRF-1. In order to map the IRF-1-interaction domain on DMP1, a series of deletion proteins was tested for their ability to interact with IRF-1. Similar to the results with IRF-4, the DNA binding domain (238-458) of DMP1 was sufficient to mediate this interaction.

Recent studies have further established the role of IRF-1 as a tumor suppressor. IRF-1 expression reverts the tumorigenic phenotype exerted by the c-myc and fosB oncogenes[21]. The *IRF-1* gene maps to chromosome 5q31.1, a region that was consistently deleted at one or both alleles in each of 13 cases of leukemia and preleukemic myelodysplasia[22]. Furthermore, bone marrow and peripheral mononuclear cells from patients with myelodysplastic syndrome (MDS) or leukemia secondary to MDS preferentially express an exon-skipped *IRF-1* mRNA, which lacks exons 2 and 3[23, 24]. The tumor suppressor function of IRF-1 was further assessed in IRF-1 knockout mice. Cells from mice deficient in IRF-1 are susceptible to transformation by the ras oncogene. The *DMP1* gene is located on chromosome 7q21, which is often deleted as a part of the 7q-minus and monosomy 7 abnormalities characteristic of human acute myelocytic leukemia and MDS. When DMP1-null MEFs were infected with viruses encoding oncogenic Ha-Ras, they formed numerous transformed foci[25]. IRF-1 and DMP1 are important genes that are involved in the proliferation and differentiation of normal cells. Our data suggest that both proteins interact with each other, and together they may control the expression of genes involved in cellular proliferation and differentiation. IRF4 is constitutively expressed in adult T-cell leukemia (ATL)-derived cell lines, which were infected with human T-cell leukemia virus type-I[26, 27]. The relationship between IRF-4 expression and oncogenicity is further highlighted by the observation of chromosomal translocations at (6; 14) (p25; q32), in some patients with multiple myeloma. The translocation juxtaposes the immunoglobulin heavy-chain *(IgH)* locus to *MUM1* (multiple myeloma oncogene 1); the *MUM1* locus at 6p25 is virtually identical to *IRF-4*. As a result, the *IRF-4* gene is overexpressed. IRF-4 was suggested to be involved in tumorigenesis, because the overexpression of IRF-4 caused the transformation of Rat-1 fibroblasts *in vitro*[28].

Since DMP1 could bind to IRF-1 and IRF-4 *in vitro*, we reasoned that DMP1 could bind IRF family members *in vivo* as well. However, we could not detect the association of either IRF-1 or IRF-4 with DMP1 *in vivo*. Given the co-stimulatory activity of DMP1 on the IRF target gene *in vivo*, we could exclude the possibility that we merely detected an artifactual association of DMP1 *in vitro*. Instead, our inability to detect *in vivo* association could be due to the lack of suitable antibodies against IRF members or DMP1. Another possibility is the presence of another associated protein that hinders the accessibility of antibodies to the IRF-DMP1 complex *in vivo*. Since the importance of IRF family members in many biological settings is gaining recognition, the factors that associate with and modify IRF members should be elucidated in the near future. We suggest that DMP1 is one of the indispensable factors involved in the function of IRF family members.

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