RFX1 Mediates the Serum-induced Immediate Early Response of Id2 Gene Expression*

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Id2, a negative regulator of basic helix-loop-helix transcription factors, is involved in regulating cell differentiation and proliferation. To obtain insight into the role of Id2 in cell cycle control, we investigated the mechanisms underlying the immediate early response of Id2 expression to serum stimulation in NIH3T3 cells. Luciferase reporter analysis with deletion and point mutants demonstrated the serum response element of Id2 (Id2-SRE) to be a consensus binding site for RFX1 (regulatory factor for X-box 1) present 3.0 kb upstream of the transcription initiation site of Id2. Gel shift and chromatin immunoprecipitation assays confirmed the binding of RFX1 to Id2-SRE in vitro and in vivo, respectively. In both assays, RFX1 binding was observed not only in serum-stimulated cells, but also in serum-starved cells. Knockdown of RFX1 by RNA interference disturbed the immediate early response of Id2 expression in cells and abrogated the Id2-SRE-mediated induction of luciferase activity by serum. These alterations were rescued by the introduction of RNA interference-resistant RFX1 into cells. On the other hand, in the Id2-SRE-mediated reporter assay, RFX1 with an N-terminal deletion abrogated the serum response, whereas RFX1 with a C-terminal deletion enhanced the reporter activity in serum-starved cells. Furthermore, HDAC1 was recruited to Id2-SRE in serum-starved cells. These results demonstrate that RFX1 mediates the immediate early response of the Id2 gene by serum stimulation and suggest that the function of RFX1 is regulated intramolecularly in its suppression in growth-arrested cells. Our results unveil a novel transcriptional control of immediate early gene expression.

Id (inhibitor of DNA binding/differentiation) proteins, bearing a helix-loop-helix motif as their functional domain but lacking a DNA-binding domain, are negative regulators of basic helix-loop-helix transcription factors (1, 2). Four members (Id1–Id4) constitute the protein family in mammals and play important roles in controlling cell fate determination, differentiation, and proliferation of various cell types in vitro and in vivo (1, 2). In accord with their cell cycle stimulatory activity, Id proteins are considered to be required for the G_{1}/S transition in the cell cycle (3, 4), and Id2-deficient mice exhibit a severe proliferation defect of mammary epithelial cells during pregnancy (5). In addition, overexpression of Id genes and/or proteins has been reported in various human malignancies (1, 2). In growth-arrested fibroblasts, in which Id mRNAs are barely detectable, mitogenic stimulation rapidly induces expression of Id genes (3, 6, 7). It has been reported that Id2 and Id3 are categorized into a group to which c-myc belongs in terms of the kinetics of mRNA expression in human fibroblasts induced by serum stimulation (8). In fact, Id3 was identified as an immediate early gene by differential screening (6). However, the exact mechanisms underlying Id gene induction upon mitogenic stimulation remain unclear, although Egr-1 (early growth response 1), an immediate early gene, has been shown to regulate the expression of Id1 (9) and Id3 (10).

Immediate early genes are those rapidly induced by growth stimulation without de novo protein synthesis (11, 12). Because many of them encode transcriptional regulators or secreted signaling molecules, they are thought to be involved in gene activation cascades required for the progression of the cell cycle (13). Elucidation of the induction pathways of immediate early genes can advance our understandings of how cell proliferation is controlled and how tumors develop. Immediate early genes are subclassified into genes with fast and slow kinetics of induction after mitogenic stimulation (12). Examples of immediate early genes with fast and slow kinetics are c-fos and c-myc, respectively. c-fos mRNA exhibits its peak of expression at ~0.5–1 h after growth stimulation and returns to the base line within 2 h (14), whereas c-myc expression peaks at ~1–3 h after stimulation (15). Various transcription factors are known to be critical for inducing the expression of immediate early genes after mitogenic stimulation. Examples include the serum response factor and ternary complex factors such as Elk-1 (16).

Although the induction mechanisms of several immediate early genes have been extensively studied, numerous immediate early genes have been reported, and the mechanisms of their transcriptional regulation largely remain to be determined.

Here we report that the rapid induction of Id2 gene expression in murine fibroblasts by serum stimulation is mediated by RFX1 (regulatory factor for X-box 1). RFX1 is a transcription...
factor involved in the induction of gene expression of viruses such as human hepatitis B virus and cytomegalovirus (17) and the suppression of cellular genes such as c-myc (18) and the proliferating cell nuclear antigen (19). Our work reveals a novel molecular mechanism underlying the regulation of immediate early gene expression.

EXPERIMENTAL PROCEDURES

Plasmids—The plasmid encoding the human RFX1 cDNA, pSG5-HARFX1, and its N- and C-terminal deletion constructs, RFX1ΔN(Δ1–415) and RFX1ΔC(Δ603–979), were provided by Dr. Y. Shaul (Weizmann Institute of Science, Rehovot, Israel) (20). Plasmids pDNA3-FLAG-RFX1, pDNA3-HA-RFX2, and pSG5-RFX3 were provided by Drs. A. Iwama (Chiba University, Chiba, Japan) and Z. Sun (Harvard Medical School, Boston, MA) (21, 22). A series of luciferase reporter plasmids were constructed by inserting the appropriate genomic fragments derived from pBS-Id2-N10 (23) and/or pGL2-Id2/C (24) into pGL4.12, encoding the fast response luciferase (Promega Corp., Madison, WI). The restriction sites used were BamHI, PstI, and EcoRI in pBS-Id2-N10 and KpnI, BglII, and NcoI in pGL2-Id2/C and pGL4.12. If necessary, restriction fragments were subcloned after blunting. The resultant plasmids contained the mouse genomic fragments of the Id2 gene locus as follows: pGL4-Id2-1, 4596 bp, −4516 to +80; pGL4-Id2-2, 2350 bp, −2270 to +80; pGL4-Id2-3, 1120 bp, −1040 to +80; pGL4-Id2-4, 2246 bp, −4516 to −2271; pGL4-Id2-5, 780 bp, −4516 to −3737; pGL4-Id2-6, 1466 bp, −3736 to −2271; pGL4-Id2-7, 776 bp, −3736 to −2961; pGL4-Id2-8, 142 bp, −2960 to −2819; and pGL4-Id2-9, 548 bp, −2818 to −2271. The positions were numbered from the transcription initiation site of the Id2 gene. Site-specific and deletion mutants of pGL4-Id2-8 were generated by PCR-mediated mutagenesis with the appropriate oligonucleotides following standard protocols, and the authenticity of plasmids was confirmed by sequencing.

Cell Culture and Transfection—NIH3T3, HeLa, and TIG3 (human diploid fibroblasts; Japanese Collection of Research Bioresources, Tokyo, Japan) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS; MP Biomedicals, Eschwege, Germany), 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C in a 5% CO2 humidified atmosphere. For synchronization of the cell cycle, cells were serum-starved by culture in DMEM containing 0.2% FBS for 48 h. Cycloheximide was purchased from Sigma. More than 85% of the serum-starved cells were confirmed to be in G0 or G1 phase by flow cytometry. To stimulate cells with serum, the low serum medium was changed to DMEM containing 20% FBS, and cells were maintained in this medium until used. DNA transfection was carried out using TransIT reagent (Mirus Bio Corp., Madison, WI) according to the manufacturer’s instructions.

Flow Cytometry—Cells were collected by trypsinization, fixed with 70% ethanol at −20 °C for 1 h, and incubated with 25 μg/ml propidium iodide (Sigma) and 100 μg/ml RNase in phosphate-buffered saline supplemented with 0.1% bovine serum albumin for 20 min. The cells were then analyzed using a FACSCalibur (BD Biosciences) with ModFit LT3 software.

Northern Blot Analysis—Total RNA was extracted from cultured cells using an RNeasy kit (Qiagen Inc., Valencia, CA). Five microliters of total RNA were separated by electrophoresis on a formaldehyde-containing 1.0% agarose gel, transferred onto a nylon filter, and cross-linked by UV irradiation. Radioactive DNA probes for Id2, c-myc, and Rfx1 and 36b4 rRNA were prepared by random primer labeling of the respective cDNAs with [α-32P]dCTP. Hybridization and washing were performed according to standard procedures. Hybridized filters were exposed to a BAS-2000 imaging plate (FujiFilm, Tokyo, Japan) to quantify the radioactivity. 36b4 rRNA served as a loading control.

Luciferase Reporter Assay—NIH3T3 cells were plated in a 24-well plate at a density of 1 × 104 cells/well. After 24 h, cells were transfected with reporter plasmids for 4 h, and serum starvation was started by changing the medium to 0.2% FBS-containing DMEM. After serum starvation for 48 h, cells were stimulated with serum by changing the low serum medium to 20% FBS-containing DMEM. Before and after serum stimulation, cell lysates were prepared, and their luciferase activities were measured as described previously (26) using the Dual-Luciferase reporter assay system (Promega Corp.) according to the manufacturer’s instructions with a Luminesencer PSN (Atto Corp., Tokyo, Japan). The Renilla luciferase plasmid pRL-TK (Promega Corp.) was cotransfected as an internal control. The magnitude of the serum response was determined by calculating the n-fold induction of luciferase activity before and after ...
4 h after serum stimulation. Assays were independently carried out at least three times in triplicate. The coefficients of variation (defined as the ratio of the S.D. to the mean) of luciferase activities in arrested cells were <0.3, indicating that the luciferase activities in arrested cells were accurately and reproducibly measured.

Data Analysis—Data are expressed as the means ± S.E. Differences between groups were analyzed using either Student’s t test or one-way analysis of variance, followed by the Student-Newman-Keuls test. p < 0.05 was considered statistically significant.

Electrophoretic Mobility Shift Assay (EMSA)—Preparation of nuclear extracts of NIH3T3 cells and EMSA were done as described previously (24). The protein content was determined by the Bradford method using bovine serum albumin described previously (24). The protein content was determined by the Bradford method using bovine serum albumin.

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay was done as described previously (24, 28). In brief, NIH3T3 cells were cross-linked with 1.0% formaldehyde in the medium for 2 min at 37 °C, rinsed twice with ice-cold phosphate-buffered saline, resuspended in 1.0 ml of SDS lysis buffer (28), and incubated on ice for 10 min. Cell lysates were then sonicated with a Bioruptor (Cosmo Bio Co., Ltd., Tokyo, Japan), and the sonicated lysates were precleared as described (24, 28). Immunoprecipitation was performed with 5 μg of anti-RFX1, anti-HDAC1, or anti-CAAT/enhancer-binding protein-β (C/EBPβ) polyclonal (C-19; Santa Cruz Biotechnology, Inc.) antibody at 4 °C for 1 h and then incubated with protein A- or G-Sepharose (GE Healthcare) at 4 °C for 3 h with rotation. The precipitated immunocomplex was released by boiling in 2× SDS electrophoresis sample buffer and subjected to Western blot analysis as described above.
Id2 is an Immediate Early Response Gene

Id2 gene expression in a murine fibroblast cell line (NIH3T3) during cell cycle progression. To obtain a synchronized cell population, NIH3T3 cells were serum-starved with 0.2% FBS-containing DMEM for 48 h and then stimulated with 20% FBS-containing DMEM. RNA was prepared before and after the stimulation and subjected to Northern blot analysis. Id2 mRNA was undetectable in a growth-arrested state and was induced as early as 30 min after serum stimulation (Fig. 1A) as reported previously (7). Id2 and TE (Tris/EDTA) buffer. Immunocomplexes were eluted with elution buffer, and reversal of cross-linking and purification of DNA were done as described (28). Precipitated DNA was resuspended in 100 µl of TE buffer for input DNA and 20 µl of TE buffer for ChIP DNA. The DNA fragments obtained were analyzed by PCR using the following primer pair: Id2, 5′-CTTTTGCCTTTCAGGGTCT-3′ or 5′-CTTGCGAGCATGAC-3′ (forward) and 5′-CCCTGACGCCTTCTC-3′ (reverse). The ChIP assay of C/EBPβ was performed as described previously (24) and used as a positive as well as a negative control. Quantitative real-time PCR for ChIP was performed using SYBR Premix Ex Taq (TaKaRa, Ohtsu, Japan) on an ABI PRISM 7000 sequence detection PCR machine (Applied Biosystems, Foster City, CA).

RNA Interference—RNA interference was carried out by introducing pSUPER.puro-RFX1-1 or pSUPER.puro-RFX1-2 into NIH3T3 cells. In Northern and Western blot analyses, an siRNA-expressing cell population was enriched by culturing transfectants with 5 µg/ml puromycin (Sigma) for 2 days. NIH3T3 cells constitutively expressing RFX1-siRNA1 or RFX1-siRNA2 were established by puromycin selection for 7 days after transfection with pSUPER.puro-RFX1-1 or pSUPER.puro-RFX1-2, respectively, and maintained with the drug.

Immunocytochemistry—Cells were fixed with 4% formaldehyde, stained with anti-hemagglutinin monoclonal antibody 16B12 (CRP Inc., Denver, PA) and then with Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes), and mounted with a mounting medium containing 4′,6-diamidino-2-phenylindole dihydrochloride (Vector Laboratories, Burlingame, CA). The cells were observed with an Olympus inverted fluorescence microscope.

RESULTS

Id2 Is an Immediate Early Response Gene—We examined Id2 gene expression in a murine fibroblast cell line (NIH3T3) during cell cycle progression. To obtain a synchronized cell population, NIH3T3
mRNA was induced in a biphasic pattern with peaks at 4 and 14–16 h after serum stimulation, similar to Id2 expression at the mRNA level in human primary cultured fibroblasts (8) and in human diploid fibroblast TIG3 cells (3). To determine whether serum-induced Id2 expression depends on de novo protein synthesis, we treated NIH3T3 cells with cycloheximide, an inhibitor of protein synthesis, and analyzed Id2 mRNA expression after serum stimulation by Northern blotting. As shown in Fig. 1B, cycloheximide did not interfere with serum-induced Id2 mRNA expression and rather augmented it at 30 min after serum stimulation. Similar results were obtained with TIG3 cells (data not shown). These results indicate that Id2 is one of the immediate early genes that are rapidly induced in response to growth stimulation without de novo protein synthesis.

**Induction of Id2 Gene Expression by Serum Is Dependent on RFX1**—To explore the molecular basis of the immediate early response of Id2 to serum stimulation, we tried to identify the genomic region that confers the serum response using a luciferase reporter assay. To this end, the upstream genomic fragment of the mouse Id2 gene was subcloned into the pGL4.12 reporter plasmid, which encodes a degradation-prone luciferase protein, enabling us to trace a rapid change in reporter activity. After transfection of the reporter plasmid pGL4-Id2-1, into NIH3T3 cells, the cells were serum-deprived with 0.2% FBS-containing DMEM for 48 h and then stimulated with 20% FBS-containing DMEM for 4 h. The extent of serum-induced transcription was evaluated by calculating the fold induction of the luciferase activity before and after serum stimulation (luciferase activity at 4 h/luciferase activity at 0 h). As shown in Fig. 2A, the 4.6-kb DNA fragment upstream of the Id2 gene exhibited an ability to induce 5.5-fold induction of the luciferase activity, suggesting that this region contains an element responsive to serum. We then constructed a series of deletion mutants of this genomic region and analyzed their reporter activities. As a result, a 142-bp region flanked by PstI sites, located 3.0 kb upstream of the transcription initiation site of mouse Id2, was found to be sufficient to induce the serum response (pGL4-Id2-8 in Fig. 2A), although a minor contribution of the proximal 2350-bp region was also noted. The 142-bp serum-responsive region contains potential binding sites for a basic helix-loop-helix factor, cAMP response element-binding protein, cut-like homeodomain protein, NF-E2 (nuclear factor erythroid 2), RFX1 and p53 and is highly conserved between humans and mice (Fig. 2B). To specify which transcription factor is involved in the serum response of Id2, we introduced site-directed mutations into each potential binding site in the region and measured the resultant reporter activities before and after serum stimulation. As shown in Fig. 2C, the reporter bearing a mutation in the potential binding site for RFX1 displayed virtually no serum response, in contrast to the lack of a drastic alteration in the serum response with the mutants of the other consensus binding sites, although a slight reduction was observed in the mutants of E-box 2 and of the potential binding sites of NF-E2 and p53. In accordance with this, deletion of the Id2 reporter plasmid containing the luciferase reporter element of Id2 (Id2-SRE) to be the potential binding site for RFX1.

**RFX1 and Immediate Early Response of Id2**

![Figure 2. Reporter assay for Id2-SRE.](image)

- A, the structure of the 4596-bp genomic fragment upstream of the Id2 gene (−4516 to −80 nucleotides from the transcription initiation site of Id2) is illustrated at the top with the restriction sites (BamHI (B), EcoRI (E), NotI (N), and PstI (P)). Luciferase (LUC) reporter plasmids used in this assay are schematically presented on the left. Thick lines indicate the locations of the genomic fragments examined, and their sizes are shown above the lines. The positions of the fragments in the genomic DNA are described under “Plasmids” under “Experimental Procedures.” The names of the reporter plasmids are shown in the middle. After transfection of a reporter plasmid into NIH3T3 cells, luciferase activities before and 4 h after serum stimulation were determined, and the fold induction of the reporter activity was calculated. The results are presented as the means ± S.E. on the right and are representative of at least three independent experiments with triplicate assays. Asterisks indicate statistically significant reduction of the reporter activity compared with that of pGL4-Id2-1 (**, p < 0.01). B, shown is the sequence of the 142-bp serum-responsive region of mouse Id2 and its comparison with the human counterpart. Potential binding sites for transcription factors in this region are boxed (E-box (the binding site for the basic helix-loop-helix factor), CAMP response element-binding protein (CREB), cut-like homeodomain protein (CDP), NF-E2, RFX1, and p53). The positions of nucleotides were calculated based on the sequences reported in the GenBank™ Data Bank (mouse accession number NT039548; and human, accession number NT005334) relative to the respective transcription initiation sites. Asterisks indicate nucleotides conserved between humans and mice. C, shown are the results from site-specific mutation analyses of the 142-bp serum-responsive region of Id2. Schematic representations of reporter plasmids and their fold inductions of luciferase activities before and 4 h after serum stimulation are shown on the left and right, respectively. × and √, substitution mutations (mut) and deletion (del) of potential binding sites for transcription factors, respectively. The names of reporter plasmids are shown in the middle. The results are presented as the means ± S.E. and are representative of at least three independent experiments with triplicate assays. Asterisks indicate statistically significant reduction of the reporter activity compared with that of pGL4-Id2-8 (**, p < 0.01; ***, p < 0.001).
in NIH3T3 cells (data not shown). RFX1 isoforms other than RFX1 therefore seem not to be involved in complexes formed with Id2-SRE. Furthermore, in vitro translation products of the human RFX1 cDNA in rabbit reticulocyte lysate formed DNA-protein complexes in EMSA, similar to those in NIH3T3 cell lysates (Fig. 3B). These results suggest that the RFX1 homodimer binds Id2-SRE, irrespective of cell conditions, and that the RFX1 monomer appears to be related to cell conditions associated with serum starvation.

To confirm the in vivo binding of RFX1 to endogenous Id2-SRE, we carried out ChIP assay with asynchronous, serum-starved, and serum-stimulated NIH3T3 cells. With chromatin fractions of serum-stimulated and asynchronous cells, anti-RFX1 antibody enriched chromatin-associated DNA containing Id2-SRE (Fig. 3C, lanes 1 and 3), similar to the result obtained for C/EBPβ (lane 10), which is a direct upstream factor of Id2 (24), whereas normal IgG did not (lanes 4–6). The binding of RFX1 to the region containing Id2-SRE was also observed with serum-starved cells (Fig. 3C, lane 2). These results indicate that RFX1 resides on genomic Id2-SRE irrespective of whether cells are growth-arrested or proliferating.

Reduction of RFX1 Leads to the Impaired Immediate Early Response of the Endogenous Id2 Gene—We examined the role of RFX1 in serum-induced Id2 gene expression by utilizing RNA interference-mediated gene suppression. For this, two siRNAs (RFX1-siRNA1 and RFX1-siRNA2) were designed according to a previous report (25), and the respective short hairpin RNA vectors were constructed based on pSUPER.puro. Their efficacies were confirmed at the RNA and protein levels by transiently expressing them in NIH3T3 cells. As shown in Fig. 4A, RFX1-siRNA1 was effective in reducing RFX1 to one-fifth the level in untreated cells, but RFX1-siRNA2 was not. We next established NIH3T3 cells harboring the respective short hairpin RNA vectors by selection with puromycin and investigated the effect of reduced RFX1 on endogenous Id2 mRNA expression by performing Northern blotting before and after serum stimulation.
RFX1 and Immediate Early Response of Id2

In cells with RFX1-siRNA1, the induction of Id2 mRNA at 0.5–12 h after serum stimulation was suppressed to be less than half compared with that observed in cells expressing non-effective RFX1-siRNA2, but the subsequent expression was not altered (Fig. 4B). In contrast, c-myc mRNA became detectable even before serum stimulation, and its expression was enhanced by a maximum of 2-fold until 4 h after serum stimulation (Fig. 4B), consistent with the report that RFX1 negatively regulates c-myc expression (18). These results demonstrate that Id2 mRNA expression is regulated positively by RFX1 during serum-induced re-initiation of growth.

Perturbed Id2 Expression in RFX1 Knockdown Cells Is Rescued by siRNA-resistant RFX1—To exclude the possibility that the phenotypes observed in RFX1 knockdown cells were derived from cross-suppression of other genes, we generated a plasmid (pSG5-HARFX1-Rs) encoding an RFX1 cDNA that was resistant to RFX1-siRNA1, but preserved the amino acid sequence of the wild-type RFX1 protein by site-directed mutagenesis as shown in Fig. 5A. After confirming that siRNA-resistant RFX1 was able to restore the level of RFX1 protein in cells expressing RFX1-siRNA1 (Fig. 5B), we performed a reporter assay and Northern blotting to examine its effect. In the reporter assay, the serum-induced luciferase activity in cells with RFX1-siRNA1 showed 2-fold reduction compared with that in parental NIH3T3 cells, whereas the reporter activity before serum stimulation seemed to be

FIGURE 3. EMSA for Id2-SRE. A, binding of RFX1 to Id2-SRE. EMSA was carried out with [α-32P]dCTP-labeled oligonucleotide probes. Nuclear extracts prepared from asynchronous cells (AS), serum-starved cells (SS), and cells at 4 h after serum stimulation (S4) were mixed with the [α-32P]dCTP-labeled oligonucleotide probe containing wild-type (W) or mutant (M) Id2-SRE. In competition experiments, the unlabeled wild-type Id2-SRE oligonucleotides (lanes 4–6) and unlabeled oligonucleotide derived from an MHC class II gene (lanes 13–15) were included. The specific DNA-protein complexes (C1 and C2) are indicated by arrowheads. Non-specific (NS) complexes and free probes are shown by open arrowheads and a bracket, respectively. The arrow points to complexes supershifted by anti-RFX1 antibody (Ab). B, DNA-protein complexes formed on Id2-SRE contain a monomer and homodimer of RFX1. EMSA was done with the [α-32P]dCTP-labeled wild-type Id2-SRE oligonucleotide probe. The antibody against RFX2 (lanes 4 and 6), RFX3 (lanes 7 and 9), RFX5 (lanes 10 and 12), or RFX1 cDNA that was resistant to RFX1-siRNA1 (lanes 13 and 14) was included in the reaction mixture. The antibody against RFX1 was used in lanes 13 and 14 instead of nuclear extracts of NIH3T3 cells. C, ChIP assay for RFX1. The chromatin prepared from asynchronous cells, serum-starved cells, and cells at 4 h after serum stimulation were precipitated with anti-RFX1 antibody (lanes 1–3 and 7–9), normal rabbit IgG (lanes 4–6), or anti-CEBP/β antibody (lane 10), and DNA purified from them was amplified with a primer set for Id2-SRE (lanes 1–6) or for the CEBP/β response element of Id2 (lanes 7–10). ChIP for CEBP/β with anti-RFX1 antibody (lanes 7–9) and anti-CEBP/β antibody (lane 10) served as negative and positive controls, respectively. DNA prepared from chromatin before ChIP was amplified by PCR with the respective primer sets, and 10% of the products are shown as input.
RFX1 and Immediate Early Response of Id2

A. The nucleotide sequences of the RFX1-siRNA1 target site and its resistant RFX1 mutant. The position of the nucleotide in the RFX1 cDNA and the encoded amino acid residues are shown on the right and top, respectively. Mutated nucleotides in the siRNA-resistant RFX1 mutant are indicated by asterisks. B. Confirmation that RFX1-siRNA1 does not target the RFX1 mutant encoded by pSG5-HARFX1-Rs. After transfection of NIH3T3 cells with the plasmids indicated at the bottom (wild-type (W), mutant resistant to RFX1-siRNA1 (Rs), and pSUPER.puro-RFX1-1 (RFX1-siRNA1)), cell lysate was prepared and subjected to Western blot analysis with the antibodies indicated on the left. Anti-hemagglutinin (α-HA) antibody recognizes RFX1 encoded by pSG5-HARFX1-Rs, whereas anti-RFX1 antibody reacts with both endogenous and mutant RFX1. C. The effect of the siRNA-resistant RFX1 mutant on the reporter driven by Id2-SRE. NIH3T3 cells were transfected with pGL4.Id2-8 and the plasmids indicated at the bottom, serum-starved for 48 h, and stimulated with 20% FBS. Luciferase reporter activity was measured before (open bars) and 4 h after (closed bars) serum stimulation. Asterisks indicate significant reductions of reporter activity (** p < 0.01). D. The effect of the siRNA-resistant RFX1 mutant on Id2 and c-myc mRNA expression. NIH3T3 cells were transfected with the plasmids indicated at the bottom and serum-starved for 48 h. Four hours after serum stimulation with 20% FBS, RNA was prepared and subjected to Northern blot analysis.

slightly elevated in RFX1-reduced cells (Fig. 5C). In this experimental setting, coexpression of siRNA-resistant RFX1 restored the reporter activities before and after serum stimulation, whereas wild-type RFX1 had no apparent effect (Fig. 5C). In Northern blot analysis, Id2 and c-myc mRNA levels were reduced and enhanced, respectively, by RFX1-siRNA1 at 4 h after serum stimulation and were restored to the levels in parental NIH3T3 cells by the presence of siRNA-resistant RFX1 (Fig. 5D). These results demonstrate that RFX1-siRNA-mediated alteration of gene expression is due to the decreased RFX1 level.

N- and C-Terminal Regions of RFX1 Differentially Regulate Id2 mRNA Expression—As shown by the EMSA and ChIP analyses, in addition to its involvement in serum-induced immediate transactivation of Id2, RFX1 appears to play some role in the suppression of Id2 gene expression in growth-arrested cells. In fact, RFX1 has been reported to be a binary transcription factor: a transactivator for MHC class II (30, 31) and a repressor for c-myc (18), for example. Related to this, it has been demonstrated that the activation and repression domains are located in the N- and C-terminal regions of RFX1, respectively (32). To examine how RFX1 regulates transcription of the Id2 gene, we introduced wild-type RFX1 or its deletion mutants (20), together with pGL4.Id2-142 bearing Id2-SRE, into NIH3T3 cells and examined their effect on the reporter activity before and after serum stimulation. In cells before serum stimulation, the reporter activities of RFX1ΔN and RFX1ΔC were substantially altered to one-fourth and 6-fold that of wild-type RFX1, respectively (Fig. 6A). On the other hand, serum stimulation further induced the reporter activity in the presence of RFX1ΔC, although the extent of induction was weak, whereas serum-induced reporter activity was substantially suppressed in the presence of RFX1ΔN (Fig. 6A). As shown in Fig. 6B, the expression levels and subcellular localizations of wild-type and mutant RFX1 proteins were similar in immunocytochemical analyses, although RFX1ΔC showed cytosolic distribution in a fraction of the transfectants. In addition, cotransfection experiments demonstrated that wild-type RFX1 was localized in the nucleus irrespective of the presence of the RFX1 deletion mutants (data not shown). Therefore, the results with RFX1ΔN demonstrate that RFX1 is involved in serum-induced Id2 expression through its N-terminal domain, whereas those with RFX1ΔC suggest that the C-terminal domain of RFX1 is required to suppress Id2 expression during serum starvation.

HDAC1 Interacts with RFX1 and Is Recruited to Id2-SRE during Serum Starvation—Although a coactivator of RFX1 is not known, RFX1 has been reported to interact with HDCA1 and to suppress the expression of collagen α2(I) (33). To examine whether HDAC1 is also involved in the suppression of Id2 mRNA expression during serum deprivation, we first performed immunoprecipitation and Western blot analyses with cell lysates prepared from NIH3T3 cells before and 4 h after serum stimulation. As shown in Fig. 7A, the physical interaction between HDAC1 and RFX1 was detected in the lysate of serum-starved cells, whereas the interaction was much weaker in the lysate prepared from serum-stimulated cells. Next, we carried out quantitative PCR after ChIP assay to evaluate the recruitment of HDAC1 to endogenous Id2-SRE. At 24 h after serum starvation, anti-HDAC1 antibody enriched chromatin-associated DNA containing Id2-SRE to an extent similar to that observed at 48 h after serum starvation (Fig. 7B). The enrichment was reduced as early as 30 min after serum stimulation, and the situation continued afterward (Fig. 7B). The enrichment of chromatin-associated DNA was not detected with normal goat IgG or with a primer set for the C/EBPβ-binding site in the Id2 promoter (Fig. 7B). These results demonstrate that HDAC1 is recruited to Id2-SRE during serum starvation and suggest that there is an inverse correlation between the recruitment of HDAC1 to Id2-SRE and Id2 mRNA expression.
We have demonstrated here that Id2, a negative regulator of basic helix-loop-helix factors, is an immediate early gene and that its rapid induction in growth-arrested NIH3T3 cells in response to serum stimulation is dependent on RFX1. This is the first indication that RFX1 plays a role in the induction of immediate early gene expression. Our results reveal that RFX1 regulates Id2 gene expression in two manners: cell context-dependent and -independent manners. Cell context-dependent regulation is observed in the difference before and after serum stimulation. It is associated with a functional difference in the N- and C-terminal domains of RFX1, and the N-terminal domain of RFX1 is essential for the induction of Id2 gene expression. Furthermore, the suppression of Id2 gene expression is associated with the recruitment of HDAC1 to Id2-SRE. On the other hand, comparison of the effect of RFX1 on Id2 expression with that on c-myc expression revealed cell context-independent regulation. RFX1 differently regulates gene expression, depending on its target genes, even under the same cell conditions.

RFX1 was first identified as a factor that binds the X-box present in the regulatory element of the MHC class II genes (30, 31) and was subsequently found to be identical to a factor that binds cis-regulatory elements present in the enhancers of several viruses, including human hepatitis B virus (17). It is characterized by a winged helix-type DNA-binding domain with a unique binding property (34) and constitutes the RFX family together with four structurally related factors (RFX2–RFX5) in mammals (22, 35–37). RFX1 displays a binary function as a transcription factor: it activates expression of the interleukin-5 receptor-α gene (21) and the neuronal glutamate transporter type 3 gene (38), whereas it suppresses expression of several genes such as c-myc (18), proliferating cell nuclear antigen (19), collagen α(1) (33, 39), and RFX1 (40). Analysis of the structure-function relationship of RFX1 has revealed that the activation and repression domains are present in the N- and C-terminal domains of RFX1, respectively (32).

The expression level and subcellular localization of the RFX1 protein are not changed in NIH3T3 cells before and after serum stimulation, although it was reported that RFX1 translocates to the nucleus upon activation of protein kinase C in HL-60 leukemia cells (41). In addition, irrespective of the cell context, ChIP assay revealed the loading of RFX1 on the 5′-flanking sequence of the Id2 gene, and gel shift assay demonstrated that the RFX1 homodimer is a major complex formed with Id2-SRE. These results suggest that RFX1 is always loaded on Id2-SRE and ready to induce Id2 gene expression via its N-terminal domain in response to serum stimulation. This mode of action is in accordance with the properties of immediate early genes that are induced rapidly without de novo protein synthesis upon stimulation. It has been postulated that RFX1 behaves as a nearly inactive transcription factor at certain times because of mutual neutralization by the activation and repression domains of RFX1 and that relief of its neutralization allows RFX1 to serve as an activator or a repressor (32). On the basis of this notion, we speculate that serum stimulation leads to the functional shift in RFX1 from being a “neutralized” transcription factor to being a transactivator, which explains why C-terminal deletion of RFX1 caused a substantial enhancement of Id2-SRE-mediated reporter activity in growth-arrested cells, whereas reduction of RFX1 by siRNA did not result in an apparent increase in Id2-SRE-mediated reporter activity or in endogenous Id2 mRNA expression. It is natural to think that serum-induced alteration of cell conditions leads to the modification of RFX1 and then triggers the activation of
FIGURE 7. HDAC1 interacts with RFX1 and is recruited to Id2-SRE during serum starvation. A, physical interaction of RFX1 and HDAC1. NIH3T3 cells were serum-deprived for 48 h, and cell lysates were prepared before (0) and 4 h after (4) serum stimulation. Immunoprecipitation (IP) was performed with the antibodies indicated at the top, followed by Western blot (WB) analyses with the antibodies indicated on the left. B, quantitative PCR after ChIP assay for HDAC1. The chromatin prepared from asynchronous (AS), serum-starved, and serum-stimulated cells was precipitated with anti-HDAC1 antibody or normal mouse IgG, and DNA purified from them was subjected to real-time PCR with the primer sets indicated on the right. The upper panels show the results of agarose gel electrophoresis. Quantitative results are schematically represented in the lower panels. Asterisks indicate a significant difference in the amount of amplified DNA compared with that of asynchronous cells (**, p < 0.01).

RFX1, although the precise mechanisms remain to be determined. Monomeric RFX1 found in serum-starved cells may be a reflection of such mechanisms. Alternatively, a third factor may modulate the property of RFX1 under the influence of stimuli from the outside. It is conceivable that cofactors possessing an enzymatic activity to modify histones are involved in transcriptional regulation of the genes. In the case of the c-myc gene, RFX1 interacts with c-myc intron-binding protein 1 and regulates c-myc expression by binding to the regulatory elements (18). Little is known about such factors, and the identification of factors that associate with and modulate the function of RFX1 as a transactivator would be necessary to understand the gene regulatory mechanisms. Meanwhile, although Id2 has been suggested to be a target of c-Myc (4), investigations with mice carrying the c-myc transgene and the inactivated Id2 gene have indicated that Id2 is not required for Myc-induced skin neoplasia (42) or lymphomagenesis (43). These observations are consistent with our results showing the inverse regulation of Id2 and c-myc expression by RFX1 in serum-stimulated cells.

Factors that play an important role in immediate early gene expression may still remain unidentified, as we have indicated here. Further investigation will advance our understanding of how the gene regulatory network operates in response to growth stimulation and controls cell cycle progression.

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