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Studies on the Hematopoietic Signal Pathway in Fetal Liver across Gestation

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Notch genes encode evolutionarily conserved receptors that have been utilized to control cell fate decisions during development. Notch signaling participates in several cellular functions such as proliferation, apoptosis, and differentiation, depending upon the cellular context of Notch activation. In the prevailing model for Notch signaling, Notch receptors are activated through binding with ligands on neighboring cells. Notch intracellular domains are released and translocated into nucleus after proteolytic cleavages triggered by ligand binding. Then Notch intracellular domains activate the expression of their target genes *via* both CBF1-dependent and –independent pathway.

The control of Notch signaling is very complicated and not fully understood yet. So far, there are several Notch 1 receptor intracellular domain (N1IC)-associating cellular factors that were identified to modulate the Notch signaling both positively and negatively. These data indicate that the activity of Notch signaling is modulated by different cellular factors in different subcellular compartments. Though the members of Notch-associated factors and the downstream target genes are expanding, the molecular mechanisms of Notch signaling in diverse developmental systems remain unsolved.

In the previous study, we had screened and characterized the N1IC-associating proteins. The transcription factor Ying Yang 1 (YY1) was identified to associate with N1IC in a high-molecular-weight complex in nucleus and this association modulated the CBF1-dependent gene expression. Furthermore, YY1 indirectly regulate the transcriptional activity of the wild-type CBF1 response elements *via* the direct interaction of N1IC and CBF1. We also demonstrated the association between endogenous N1IC and intrinsic YY1 in both of the human Jurkat cells and acute T cell lymphoblastic leukemia (T-ALL) cells. Taken together, these results indicate transcription factor YY1 may modulate Notch signaling *via* association with the large Notch complex.

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Association of Transcription Factor YY1 with the High Molecular Weight Notch Complex Suppresses the Transactivation Activity of Notch*

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Notch receptors are evolutionarily conserved from *Drosophila* to human and play important roles in cell fate decisions. After ligand binding, Notch receptors are cleaved to release their intracellular domains. The intracellular domains, the activated form of Notch receptors, are then translocated into the nucleus where they interact with other transcriptional machinery to regulate the expression of cellular genes. To dissect the molecular mechanisms of Notch signaling, the cellular targets that interact with Notch1 receptor intracellular domain (N1IC) were screened. In this study, we found that endogenous transcription factor Ying Yang 1 (YY1) was associated with exogenous N1IC in human K562 erythroleukemic cells. The ankyrin (ANK) domain of N1IC and zinc finger domains of YY1 were essential for the association of N1IC and YY1 according to the pull-down assay of glutathione *S*-transferase fusion proteins. Furthermore, both YY1 and N1IC were present in a large complex of the nucleus to suppress the luciferase reporter activity transactivated by Notch signaling. The transcription factor YY1 indirectly regulated the transcriptional activity of the wild-type CBF1-response elements via the direct interaction of N1IC and CBF1. We also demonstrated the association between endogenous N1IC and intrinsic YY1 in human acute T-cell lymphoblastic leukemia cell lines. Taken together, these results indicate that transcription factor YY1 may modulate Notch signaling via association with the high molecular weight Notch complex.

Notch genes encode evolutionarily conserved receptors that are utilized to control cell fate decisions during development. Notch signaling participates in several cellular functions such as proliferation, apoptosis, and differentiation, depending upon the cellular context of Notch activation (reviewed in Refs. 1–3).

Human Notch was first identified as a gene involved in the t(7;9)(q34; q34.3) chromosomal translocation detected in some T-cell acute lymphoblastic leukemias. This gene was shown to generate Notch1 receptor intracellular domain (N1IC),[1] an activated form of Notch1 receptor (4). Further studies showed that the human Notch receptor family consists of four members (Notch1–4), located on chromosomes 9q34, 1p13-p11, 19p13.2-p13.1, and 6p21.3, respectively (5, 6). Notch receptors are single-span transmembrane proteins with several functional domains. In the extracellular domain, there are several epidermal growth factor repeats and three Lin/Notch repeats. The Notch intracellular domain consists of a RAM domain, ankyrin repeats (ANK), nuclear localization signal, a transcriptional activator domain (TAD), and a proline-glutamate-serine-threonine-rich (PEST) domain. The RAM domain is the primary binding site of activated Notch receptor with C promoter binding factor-1 (CBF1)/recombination signal binding protein-Jκ (RBP-Jκ) (7), a human homolog of *Drosophila* Su(H). The ANK repeat domain can also associate with CBF1 to modulate the interaction (8).

In the prevailing model of Notch signaling, Notch receptors are activated through binding with ligands on neighboring cells. The Notch intracellular domains are released and translocated into the nucleus after proteolytic cleavages triggered by ligand binding. Then Notch intracellular domains activate the expression of their target genes via both CBF1-dependent and -independent pathways (reviewed in Ref. 9).

The control of Notch signaling is very complicated and not yet fully understood. So far, there are several N1IC-associated cellular factors that have been identified to both positively and negatively modulate Notch signaling. These identified N1IC-associated cellular factors include Numb (10, 11), CIR (12), SKIP (13), MAML1/LAG3 (14), Deltex (15, 16), CBF1/RBP-Jκ/Su(H) (7, 17, 18), EMB-5 (19), Dishevelled (20), Disabled (21), Nur77 (22), presenilin-1 (23), MEF2C (24), PCAF, GCN5 (25), SEL-10 (26), NF-κB (27), p300 (28), and LEF-1 (29). These data indicate that the activity of Notch signaling is modulated by different cellular factors in different subcellular compartments. Several downstream target genes of N1IC have been identified, including HES family (30, 31), Nrarp (32), HERP2 (33), cyclin D1 (34), AP-1 (35), pre-T-cell receptor α (pTα) gene (36), and acid α-glucosidase (37).

Although the members of Notch-associated factors and the downstream gene targets are expanding, the underlying molecular mechanisms of Notch signaling in diverse developmental systems remain unresolved. To further dissect Notch signaling, we screened and characterized activated Notch1 receptor-associated proteins in a hematopoietic system. The transcription factor Ying Yang 1 (YY1) was identified to be

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[1] The abbreviations used are: N1IC, Notch1 receptor intracellular domain; YY1, Ying Yang 1; RAM, RAM23 domain; ANK, ankyrin repeats; PEST, proline-glutamate-serine-threonine-rich; TAD, transcriptional activator domain; GST, glutathione *S*-transferase; HA, hemagglutinin; ChIP, chromatin immunoprecipitation; CAT, chloramphenicol acetyltransferase.

associated with the activated form of Notch1 receptor in a high molecular weight complex in the nucleus, and this association modulated the CBF1-dependent gene expression.

EXPERIMENTAL PROCEDURES

Plasmids and Plasmid Construction—The cDNA of Notch1 receptor intracellular domain was cloned by RT-PCR from the total RNA of HL-60 cells. The expression construct of pcDNA-HA-N1IC, a derivative of the mammalian cell expression vector pcDNA3-HA2, contains the cDNA-encoded amino acid residues 1764–2444 of human Notch1 receptor with an HA tag at the N terminus. The fusion protein plasmids GST-ANKΔEP (28), pGST-ANK, pGST-R-A, pGST-A-T, and pGST-N1IC direct the expression of GST fusion proteins with amino acid residues 1821–2095, 1821–2205, 1764–2205, 1821–2444, and 1764–2444 of human Notch1 receptor, respectively. Plasmids pGST-YY1-(1–54), pGST-YY1-(1–80), pGST-YY1-(1–154), pGST-YY1-(1–198), pGST-YY1-(1–295), pGST-YY1-(1–414), and pGST-YY1-(296–414), which encode various lengths of YY1 protein fused with GST protein, were used for GST pull-down assay. Reporter plasmids, 4×wtCBF1Luc and 4×mtCBF1Luc, contain four copies of wild-type or mutant CBF1-response elements in front of a simian virus 40 promoter-driven luciferase (39). The CAT reporter plasmid (pFPIII$_{\nu}$-CAT) contains six CBF1-binding sites fused to the thymidine kinase promoter element and bacterial *CAT* gene coding sequence (40).

Cell Culture and Transfection—The human erythroleukemia cell line K562 and acute T-cell lymphoblastic leukemia Jurkat and SUP-T1 cells were cultured in RPMI1640 with 10% fetal bovine serum. HEK293 cells were cultured in Dulbecco's modified Eagle's medium with 10% horse serum.

For the establishment of stable K562 cell lines expressing HA-N1IC (K562/HA-N1IC), K562 cells (2×10^6) were transfected with a linearized expression plasmid of pcDNA-HA-N1IC (5 μg) by electroporation using a Bio-Rad gene pulser electroporator. Forty-eight hours after electroporation, cells were diluted to about 0.8 cell/well in 96-well dishes and selected with 800 μg/ml G418. The stable clones derived from single cells were screened for the expression of HA-N1IC fusion protein by Western blot with anti-HA and anti-Notch1 C-terminal antibodies (Santa Cruz Biotechnology). For the control, the linearized pcDNA3-HA2 vector was also electroporated into K562 cells to establish a stable cell line (K562/pcDNA3).

For the CAT reporter assay, HEK293 cells (2×10^5) in 6-well culture plates were incubated with 2 ml of calcium phosphate-DNA coprecipitate containing 2.5 μg of pFPIII$_{\nu}$-CAT reporter plasmid and 2.5 μg of various expression plasmids (as indicated in the figure legends). To correct for transfection efficiency, 0.25 μg of pcDNA3.1/myc-his/LacZ was used as an internal control. Cells were harvested in the reporter lysis buffer 48 h after transfection. CAT activity was assayed as described previously (40). For the transient transfection of luciferase reporter assay, K562 cells (1×10^6) were seeded onto 6-well plates and transfected using the SuperFect transfection reagent (Qiagen). Reporter plasmids containing wild-type (4×wtCBF1Luc) or mutant (4×mtCBF1Luc) CBF1-response elements (1.0 μg) were cotransfected with pRL-TK (0.02 μg), pcDNA-HA-N1IC (1.0 μg), and pCMV-YY1 (1.0 μg) or their control vectors (3.0 μg). Forty-eight hours after transfection, luciferase activities derived from both *Firefly* (4×wtCBF1Luc and 4×mtCBF1Luc) and *Renilla* (pRL-TK) luciferase proteins were measured using the Dual-Luciferase™ reporter assay system (Promega). *Renilla* luciferase activity was then used to normalize for transfection efficiency.

For chromatin immunoprecipitation (ChIP) experiments, the K562/HA-N1IC cells (5×10^6) were transfected with 5 μg of reporter plasmids 4×wtCBF1Luc and 4×mtCBF1Luc; cells were harvested 24 h after transfection.

Coimmunoprecipitation—To prepare whole-cell lysates, cells were lysed in NETN buffer (50 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.5 mM EDTA, and 0.5% Nonidet P-40) containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 mM sodium fluoride). Protein A-Sepharose was washed with NETN buffer to obtain a 50% (v/v) slurry. Five μl of anti-YY1 (Santa Cruz Biotechnology) or anti-Notch1 C-terminal antibodies and 50 μl of 50% (v/v) slurry of protein A-Sepharose were added into 450 μl of NETN buffer and then rotated at 4 °C for more than 1 h to prepare the slurry of antibody-conjugated protein A-Sepharose. Immunoprecipitation was performed by rotating the mixture of cell lysates and mouse IgG-bound protein A-Sepharose (control) or antibody (anti-YY1 or anti-Notch1 C terminus)-conjugated protein A-Sepharose at 4 °C for more than 1 h (41). Laemmli sample buffer was added to the immunoprecipitated pellets and heated at 95 °C for 5 min and then analyzed by SDS-PAGE. The Western blot was performed with anti-Notch1 C terminus, anti-YY1, or anti-HA antibodies.

GST Pull-down Assay—*Escherichia coli* strains DH5α harboring GST and GST fusion protein expression vectors were cultured for the purification of GST and GST fusion proteins. After induction by 0.8 mM isopropyl-β-D-thiogalactopyranoside for 4 h, cells were harvested and lysed with ice-cold phosphate-buffered saline containing protease and phosphatase inhibitors and 1 mg/ml lysozyme at 4 °C for 30 min. Then Triton X-100 was added to cell suspensions to a final concentration of 0.2%, and the suspensions were rotated for another 10 min at 4 °C. The lysates were loaded onto a 50% slurry of glutathione-agarose resin, and the mixtures were gently rocked for 30 min at room temperature. The unbound proteins were decanted after centrifugation, and the resin was washed with phosphate-buffered saline several times.

Whole-cell extracts of K562/HA-N1IC and K562/pcDNA3 cells were prepared in NETN buffer as described above. To 500 μg of whole-cell extracts, 50 μl of a 50% (v/v) slurry of glutathione-agarose resin pre-bound with 0.5 μg of GST or GST fusion proteins was added and then rotated at 4 °C for 2 h. After centrifugation to remove the unbound fraction, the slurry of glutathione-agarose resin was washed by NETN buffer three times and denatured in sample buffer for SDS-PAGE and Western blot.

Subcellular Fractionation—To prepare the nuclear extracts of K562/HA-N1IC and K562/pcDNA3 cells, the cell pellets were suspended in a hypotonic buffer (20 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 10 mM KCl, 0.5% Nonidet P-40, 0.5 mM dithiothreitol, and protease and phosphatase inhibitors) at 4 °C for 30 min. After centrifugation at 4,000 × *g* at 4 °C for 10 min, the pellets of nuclei were resuspended in a high salt buffer (20 mM HEPES (pH 7.4), 0.4 M NaCl, 1 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 mM sodium fluoride) and then incubated on ice for 30 min. The supernatants recovered after centrifugation were treated as nuclear extracts.

Sucrose Gradient Analysis—Nuclear extracts (0.4 ml) of K562/HA-N1IC or K562/pcDNA3 cells were loaded on the top of 10.8 ml of a 10–60% (w/v) sucrose gradient prepared in NETN buffer. The samples were subjected to centrifugation at 38,000 rpm in a SW41 rotor (Beckman Instruments) at 4 °C for 15.5 h. The gradients were fractionated into 0.5-ml fractions each from the top, and aliquots of each fraction were subjected to immunoblotting for the detection of N1IC and YY1 proteins. The protein standards (catalase, 11.3 S, 232 kDa; thyroglobulin, 19.4 S, 669 kDa) were prepared in high salt buffer and run on a sucrose gradient. The collected fractions were assayed for protein to determine the corresponding positions of the protein standards on the gradients.

Oligoprecipitation—The 5′-biotinylated oligonucleotides with wild-type sequence (5′-AGATGCAGTCGCTGAGATTCTTTGGCCG-3′) or mutant sequence (5′-AGATGCAGTCGCCTGCAGTCTTTGGCCG-3′) of CBF1-binding sites were annealed with their complementary oligonucleotides for oligoprecipitation as described by Otsuka *et al.* (42).

Chromatin Immunoprecipitation—Transfected cells were treated with formaldehyde (1% final concentration) at room temperature for 15 min to cross-link DNA and protein, and the reaction was stopped by adding glycine to a final concentration of 0.125 M. Cells were lysed in 500 μl of NETN buffer with protease and phosphatase inhibitors. After the removal of cell debris, the supernatant was immunoprecipitated with protein A-Sepharose-bound anti-YY1 or anti-Notch1 C-terminal antibodies at 4 °C for 12–16 h. After centrifugation, the pellets were washed with NETN buffer, and the immunoprecipitates were eluted with 150 μl of elution buffer (50 mM $NaHCO_3$, 1% SDS) twice by vortexing. Then 30 μg of RNase A, 36 μl of 5 M NaCl, and elution buffer were added to a final volume of 600 μl. The cross-linking of DNA and protein in the immunoprecipitates was reversed by heating at 67 °C for 5 h. After phenol extraction, DNA was precipitated by ethanol and resuspended in 50 μl of H_2O. Five μl of each sample and control plasmid of 4×wtCBF1Luc (4 ng) were used as a template for PCR amplification using the specific primers 5′-TGTATCTTATGGTACTGTAACTG-3′ and 5′-CTTTATGTTTTTGGCGTCTTCCA-3′. At 25, 30, and 35 cycles of amplification, PCR products (5 μl) were removed and run on a 1.0% agarose gel and then analyzed by ethidium bromide staining (43).

RESULTS

Association of the Intracellular Domain of Notch1 Receptor with YY1—Expression plasmids harboring various lengths (ANK, RAM-ANK, ANK-TAD, and N1IC) of Notch1 receptor intracellular domain were transfected into HEK293 cells to

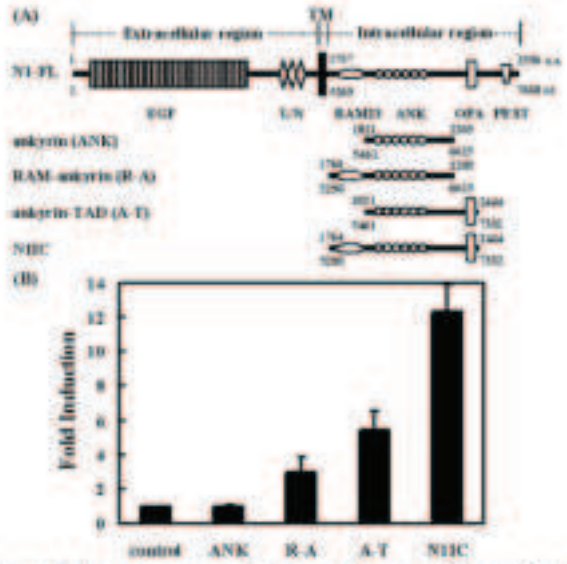

FIG. 1. **Schematic representation of human N1IC-specific expression constructs and transactivation activities of these constructs through endogenous CBF1.** *A*, the human Notch1 receptor and derived constructs containing different domains of intracellular domains were used for expression. All constructs carried an N-terminal HA tag. The first and the last amino acids and nucleotides compared with the full-length protein are indicated above and below the diagram of each construct, respectively. *B*, transcription activity of N1IC-derived constructs. The reporter construct, $p(FPIII)_4$-CAT, was cotransfected into HEK293 cells together with the indicated expression plasmids. After 48 h, CAT activity was determined from whole-cell extracts, and the basal promoter activity of the reporter construct was set to unity. Mean values and standard deviations from at least four independent experiments are shown.

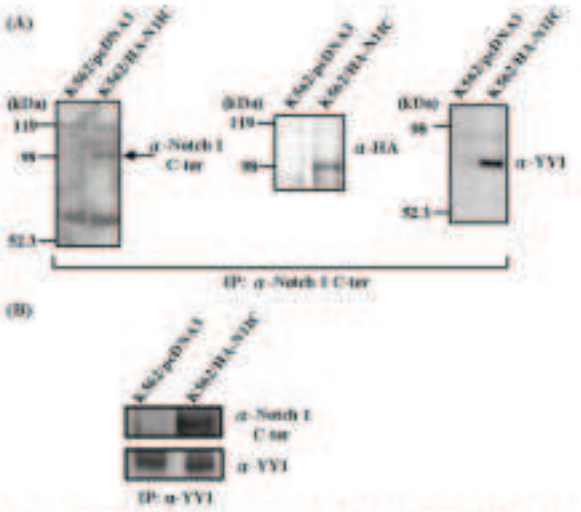

FIG. 2. **Human N1IC associated with endogenous YY1 in K562 cells.** *A*, whole-cell extracts of K562 cells (K562/pcDNA3) and HA-N1IC protein-expressing K562 cells (K562/HA-N1IC) immunoprecipitated with anti-Notch1 C-terminal antibody. The precipitated proteins were resolved by SDS-PAGE and analyzed by Western blot using anti-Notch1 C-terminal (*C-ter*) antibody (*left panel*), anti-HA antibody (*middle panel*), or anti-YY1 antibody (*right panel*). *B*, whole-cell extracts immunoprecipitated (*IP*) with anti-YY1 antibody. The precipitated proteins were resolved by SDS-PAGE and analyzed by Western blot using anti-Notch1 C-terminal antibody (*upper panel*) or anti-YY1 antibody (*lower panel*).

analyze CBF1-dependent transactivation activities by the CAT reporter assay. The expressed RAM-ANK, ANK-TAD, and N1IC, but not the ANK domain, were able to transactivate the reporter gene through the endogenous CBF1 with the highest activation by N1IC (Fig. 1). Results of the CAT assay reflect the fact that the RAM domain is the primary binding domain for CBF1, whereas ANK repeats interact weakly with CBF1, and TAD is important for transactivation activity (44).

To gain insight into the mechanism of Notch signaling, we used a yeast two-hybrid system and pull-down assay combined with proteomic analyses to identify cellular factors associated with the human-activated Notch1 receptor, N1IC. The transcription factor YY1 is one of the identified candidates of N1IC-associated proteins. To confirm the association between YY1 and N1IC, coimmunoprecipitation was applied to stable N1IC-expressing K562 cell lines. Due to the rapid turnover and/or low level expression, the expression levels of the endogenous and exogenous N1IC were too weak to be detected in K562 cells (45). The C-terminal PEST-like domain of Notch1 receptor contributes to instability and degradation of N1IC by the ubiquitin-proteasome pathway (26). Therefore, the PEST domain of Notch1 receptor was omitted from this study in order to overexpress N1IC constitutively. The HA-N1IC fusion protein of about 98 kDa was immunoprecipitated from whole-cell extracts by antibodies against N1IC (Fig. 2*A*, *left*). After stripping, the HA-N1IC fusion protein was further confirmed by anti-HA antibody (Fig. 2*A*, *middle*). Transcription factor YY1 was also detected after stripping and reprobing this immunoprecipitated blot with anti-YY1 antibody (Fig. 2*A*, *right*). Alternatively, the HA-N1IC fusion protein was also coimmunoprecipitated with cellular YY1 by anti-YY1 antibody (Fig. 2*B*). These results showed that ectopically expressed N1IC was associated with endogenous YY1 by the analysis of coimmunoprecipitation.

Mapping the Associated Regions of N1IC and YY1 in Vitro by the GST Pull-down Assay—To dissect the region(s) essential for the association of YY1 and N1IC, the *in vitro* binding properties of these two proteins were examined by the GST pull-down assay. Partially purified GST and GST fusion proteins of N1IC and YY1 were analyzed by SDS-PAGE and Coomassie Blue staining (Fig. 3). Whole-cell extracts of K562/pcDNA3 and K562/HA-N1IC cells were prepared for the pull-down assay by GST and GST fusion proteins. All fusion proteins of GST-ANKΔEP GST-ANK, GST-RAM-ANK, GST-ANK-TAD, and GST-N1IC were associated with endogenous YY1 of K562/pcDNA3 cells (Fig. 3*A*). This result shows that only the ANK domain of Notch1 receptor was sufficient to associate with YY1. Moreover, N1IC was only associated with the GST full-length YY1 fusion protein (GST-YY1, 1–414), but not with other truncated YY1 fusion proteins, *e.g.* GST-YY1-(1–54), GST-YY1-(1–80), GST-YY1-(1–154), and GST-YY1-(1–198) or GST-YY1-(1–295) (Fig. 3*B*). Therefore, the region of amino acid residues 295–414, the zinc finger domain region, of YY1 is essential for the association with N1IC.

To determine further whether the region of amino acid residues 295–414 of YY1 is sufficient to associate with N1IC, partial purified GST and GST fusion proteins were used to pull-down N1IC in the whole-cell extracts of K562/HA-N1IC cells. N1IC was only associated with the GST full-length YY1 fusion protein (GST-YY1, 1–414) but not with truncated GST-YY1-(296–414) fusion protein (Fig. 3*C*). These results suggested that the zinc finger domain region of YY1 is essential, but not sufficient, for the association with N1IC.

The Activated Notch1 Receptor Associated with YY1 in a Large Complex in the Nucleus—The intracellular domain of Notch1 receptor had been demonstrated to be associated with the transcription factor CBF1 and Mastermind-Like-1 (Maml) in an ~1.5-MDa high molecular weight complex in the nucleus (47). To elucidate whether the association between the acti-

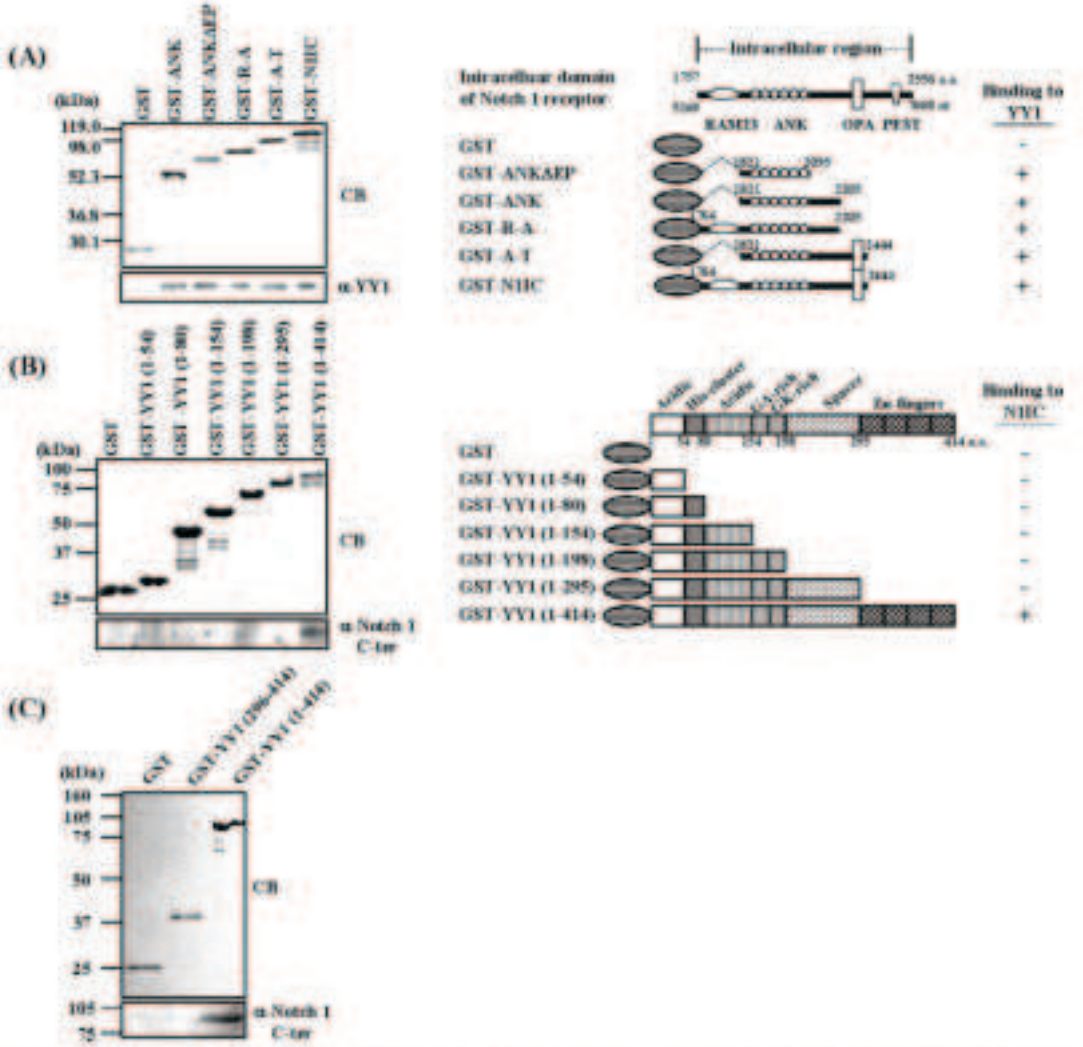

FIG. 3. **Mapping of the domains in N1IC and YY1 required for association with each other by the GST pull-down assay.** *A*, the purified GST, GST-ANKAEP, GST-ANK, GST-R-A, GST-A-T, and GST-N1IC fusion proteins (*upper portion* of *left panel*, Coomassie Blue-stained (*CB*)) used for the pull-down assay with whole-cell extracts of K562/pcDNA3 cells. The pull-down pellets were resolved by SDS-PAGE and analyzed by Western blot using anti-YY1 antibody (*lower portion* of *left panel*) and summarized the data in *right panel*. *B*, the purified GST or YY1-derived deletion GST fusion proteins (*upper portion* of *left panel*, Coomassie Blue-stained) used for the pull-down assay with whole-cell extracts of K562/HA-N1IC cells. The first and the last amino acids compared with the full-length YY1 protein are indicated in *parentheses*. The pull-down pellets were resolved by SDS-PAGE and analyzed by Western blot using anti-Notch1 C-terminal (*C-ter*) antibody (*lower portion* of *left panel*) and summarized the data in *right panel*. *C*, the purified GST, GST-YY1-(296–414), and GST-YY1-(1–414) fusion proteins (*upper panel*, Coomassie Blue-stained) used for the pull-down assay with whole-cell extracts of K562/HA-N1IC cells. The pull-down pellets were resolved by SDS-PAGE and analyzed by Western blot using anti-Notch1 C-terminal antibody (*lower panel*).

vated Notch1 receptor and YY1 occurs in the nucleus, sucrose gradient analyses were applied to nuclear extracts prepared from K562/pcDNA3 and HA-N1IC-expressing K562/HA-N1IC cells. When the collected fractions were analyzed by Western blot using anti-Notch1 C-terminal antibody, N1IC was detected in fractions 3–8 and 14–16 (Fig. 4*A*). After stripping and reprobing with anti-YY1 antibody, transcription factor YY1 was detected in fractions 1–12 and also in fractions 14–16. There were three distribution peaks of transcription factor YY1 in the sucrose gradient analysis, *i.e.* fractions 2–5, 8–10, and 14–16. The sucrose gradient analysis profile showed that N1IC and YY1 coexisted in both lower molecular weight fractions (fractions 3–5) and higher molecular weight fractions (fractions 14–16). In the absence of N1IC, YY1 showed the same sucrose gradient profile as that of K562/HA-N1IC cells. The presence of N1IC did not alter the distribution of endogenous YY1 in the sucrose gradient analysis.

To determine whether the associated complex of N1IC and YY1 is present in these coexisting fractions, fractions 3–5, 8–10, and 14–16 were individually combined and immunoprecipitated with anti-IgG or anti-Notch1 C-terminal antibodies. YY1 was coimmunoprecipitated with N1IC in fractions 3–5 and 14–16 but not in fractions 8–10 (Fig. 4*B*). This result demonstrates that N1IC is associated with YY1 as both a lower molecular weight complex (fractions 3–5) and a higher molecular weight complex (fractions 14–16) in the nucleus.

Suppression of Luciferase Reporter Activity Transactivated by Notch1 Receptor—Identification and elucidation of the physiological function of Notch receptor-associated proteins will provide insights into Notch signaling. To delineate the biological function of the association between N1IC and YY1 in Notch signaling, we used a reporter gene assay. K562 cells were cotransfected with a luciferase reporter plasmid containing four copies of wild type or mutant CBF1-response elements (4×wtCBF1Luc and 4×mtCBF1Luc, respectively), N1IC-expressing construct, pcDNA3-HA-N1IC, and YY1-expressing

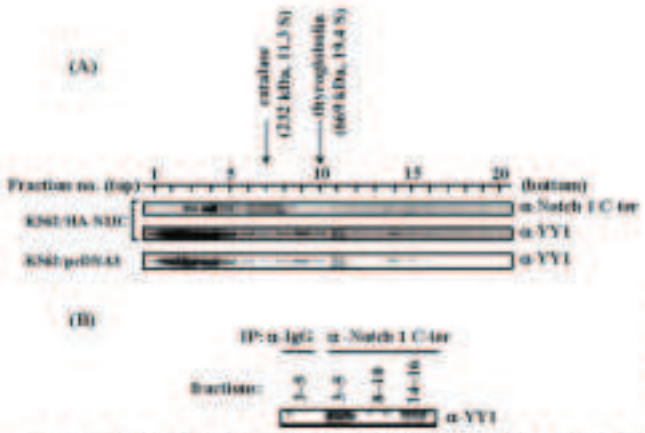

FIG. 4. **Human N1IC associated with YY1 in high molecular weight nuclear protein complexes.** *A*, nuclear extracts from K562/pcDNA3 and K562/HA-N1IC cells subjected to sucrose gradient fractionation. N1IC and YY1 were visualized by Western blot with anti-Notch1 C-terminal (*C-ter*) and anti-YY1 antibodies, respectively. *Arrows* indicate the native molecular masses of known standards. *B*, N1IC and YY1 physically associated in the low and high molecular weight complexes. Three fractions encompassing the indicated sucrose gradient peaks were pooled and immunoprecipitated (*IP*) with mouse anti-IgG or anti-Notch1 C-terminal antibody. Immunoprecipitates were detected with anti-YY1 antibody.

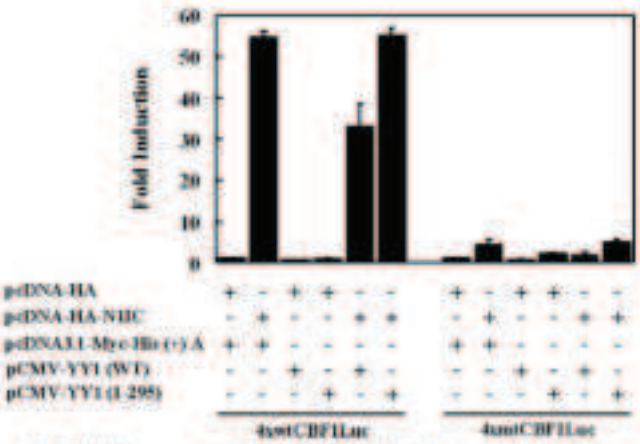

FIG. 5. **YY1 suppression of CBF1-mediated transactivation activity of N1IC.** Reporter plasmids containing wild-type or mutant CBF1-response elements were cotransfected with plasmids expressing the indicated proteins into K562 cells. After 48 h, luciferase activity was determined from whole-cell extracts, and the basal promoter activity of the reporter construct was set to unity. Mean values and standard deviations from at least four independent experiments are shown.

construct, pCMV-YY1, or their control vectors. Two days after transfection, cells were harvested and assayed for luciferase activity. Because N1IC can promote the expression of the reporter gene containing CBF1-response elements, there was a 54-fold enhancement of the luciferase activity by N1IC (Fig. 5). When cotransfected with the YY1 expression plasmid, this elevation of luciferase activity was suppressed by 39% to about 33-fold. This effect of YY1 was not observed in the luciferase reporter plasmid containing four copies of the mutant CBF1-response elements. The truncated YY1-(1–295), without the zinc finger domains, did not suppress the luciferase activity transactivated by N1IC, which also reflects the importance of the zinc finger domains in association between N1IC and YY1 as shown in Fig. 3*B*. This result implies that YY1 suppresses the luciferase reporter activity transactivated by Notch1 receptor.

Intrinsic N1IC Associated with Endogenous YY1 in Jurkat and SUP-T1 Cells—It has been reported that the activated form of Notch1 receptor is unstable and can elicit biological effects at a very low protein concentration (45). It is possible that the constitutive overexpression of N1IC may cause the fortuitous association between N1IC and endogenous YY1. To exclude this possibility, we examined the association between N1IC and YY1 in Jurkat cells by coimmunoprecipitation using anti-YY1 antibody. The intrinsic N1IC could be coimmunoprecipitated with endogenous YY1 (Fig. 6*A*). Alternatively, endogenous YY1 could also be communoprecipitated with intrinsic N1IC using anti-Notch1 C-terminal antibody (data not shown). The intrinsic YY1 could also be coimmunoprecipitated with endogenous N1IC in SUP-T1 cells (Fig. 6*B*). These results showed that intrinsic N1IC might be associated with endogenous YY1 in cells.

The YY1-N1IC-associated Complex Binds on the Wild-type CBF1-response Element but Not on the Mutant One—To prove that modulation of Notch1 signaling by YY1 was not an artifact from overexpression and/or alterations in signaling pathways, oligoprecipitation was performed to study the interaction between the YY1-N1IC-associated complex and DNA. In Jurkat cells, intrinsic N1IC and endogenous YY1 could be precipitated together with the 5′-biotinylated wild-type CBF1-response element but not the mutant one (Fig. 6*C*). However, YY1 could not be precipitated with the 5′-biotinylated wild-type CBF1-response element in K562 cells (data not shown). Additionally, the ChIP assay was also used to examine the specific association of transcription factor YY1 and N1IC with DNA in the context of living cells. Jurkat cells were transiently transfected with luciferase reporter plasmids with wild-type (4×wtCBF1Luc) or mutant (4×mtCBF1Luc) CBF1-response elements. Twenty-four hours after transfection, the cells were harvested for the ChIP assay using mouse anti-IgG, anti-YY1, or anti-Notch1 C-terminal antibodies. The amplified PCR product of 470 bp was only present in the sample prepared from wild-type CBF1-response element-transfected cells but was not present in those transfected with the mutant one (Fig. 6*D*). These data suggest that transcription factor YY1 indirectly binds on the wild-type CBF1-response element via associating with N1IC which directly interacts with CBF1. A similar result was also obtained for the ChIP assay using K562/HA-N1IC cells.

DISCUSSION

In this study, we investigated the relationship between the intracellular domain of human Notch1 receptor and transcription factor YY1. We show here that N1IC is associated with YY1 in the nucleus and that YY1 suppresses the CBF1-dependent luciferase reporter activity transactivated by Notch1 receptor. This is the first report regarding the role of the multifunctional transcription factor YY1 in CBF1-dependent Notch signaling.

Jeffries *et al.* (47) showed that a high molecular weight Notch complex of ~1.5 MDa was present in the nucleus of N1IC-transformed RKE cells and in a human T-cell leukemia cell line. We demonstrate that YY1 is associated with N1IC as both small and large complexes in the nucleus and suppresses the CBF1-dependent luciferase reporter activity transactivated by N1IC. Therefore, YY1 may modulate Notch signaling via the large high molecular weight Notch complex. In sucrose gradient analysis, the distribution profile of YY1 in the nucleus did not vary whether N1IC is presented or not. This phenomenon may be due to the multifunctionality or abundance of YY1. The ubiquitous transcription factor YY1 plays several regulatory roles in the transcription of target genes. Only a small fraction of YY1 may be involved in the regulation of Notch signaling in the nucleus. Therefore, the distribution profile of YY1 in the

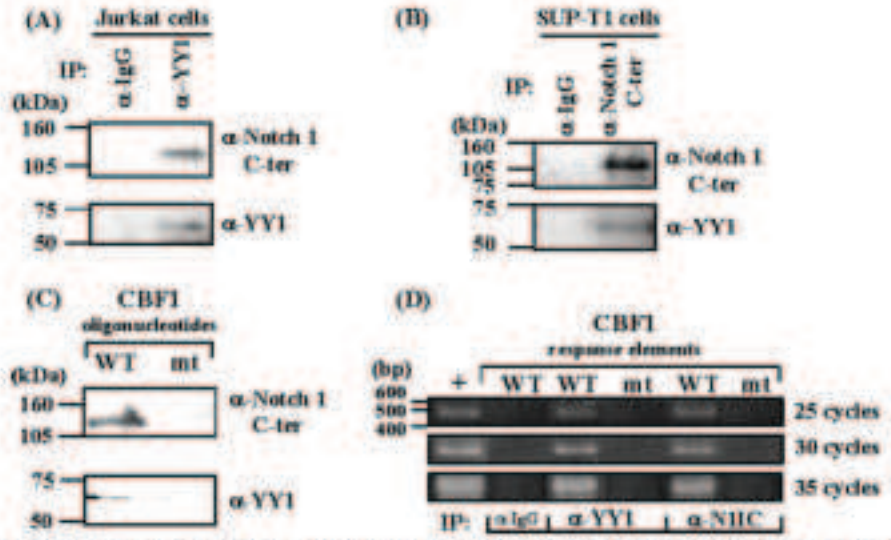

FIG. 6. **Endogenous N1IC associated with intrinsic YY1 and this complex binding to the wild-type CBF1-response element.** *A*, whole-cell extracts of Jurkat cells immunoprecipitated with anti-YY1 or anti-IgG antibody. The precipitated proteins were resolved by SDS-PAGE and analyzed by Western blot using anti-Notch1 C-terminal (*C-ter*) antibody (*upper panel*) or anti-YY1 antibody (*lower panel*). *B*, whole-cell extracts of SUP-T1 cells immunoprecipitated (*IP*) with anti-Notch1 C-terminal or anti-IgG antibodies. The precipitated proteins were resolved by SDS-PAGE and analyzed by Western blot using anti-Notch1 C-terminal antibody (*upper panel*) or anti-YY1 antibody (*lower panel*). *C*, the N1IC and YY1 complex binding to the double-stranded oligonucleotides of CBF1-response elements. Nuclear extracts of Jurkat cells were incubated with the 5′-biotinylated double-stranded oligonucleotides of wild-type (*WT*) or mutant (*mt*) CBF1-response elements and then precipitated with streptavidin-agarose beads. The precipitated proteins were resolved by SDS-PAGE and analyzed by Western blot using anti-Notch1 C-terminal antibody (*upper panel*) or anti-YY1 antibody (*lower panel*). *D*, the N1IC and YY1 complex binding to the CBF1-response elements. Jurkat cells transiently transfected with luciferase reporter plasmids containing wild-type (*WT*) or mutant (*mt*) CBF1-response elements. Twenty four hours after transfection, transfected cells were harvested for ChIP assay using mouse anti-IgG, anti-YY1, or anti-Notch1 C-terminal antibodies. The immunoprecipitated DNA was used to amplify a 470-bp PCR product by specific primers for the region of the CBF1-response element in the reporter plasmid. +, PCR-positive control uses 4 ng of 4×wtCBF1Luc plasmid as the DNA template.

absence of exogenous N1IC in the nucleus was the same as that in the presence of N1IC.

YY1 is a complex multifaceted protein that may act as a transcriptional repressor (48–50), a transcriptional activator (51), or a transcriptional initiator (52). Differential expression of the YY1 protein in myocytes affects both transcription of specific genes and cell differentiation (53). Therefore, the association of N1IC with this multifunctional transcription factor YY1 may be relevant to the diversity of Notch signaling. These results imply that YY1 may regulate cell differentiation by itself or via the control of Notch signaling.

YY1 is a ubiquitous protein abundantly expressed in both K562 and Jurkat cells. Although transient transfection of the YY1 expression plasmid showed no significant increase in the expression level of the YY1 protein in the luciferase reporter assay (data not shown), the low level of exogenous YY1 showed 39% suppression of CBF1-dependent luciferase activity transactivated by N1IC. One of the explanations is that only a small amount of exogenous YY1 is sufficient to suppress Notch transactivation. Alternatively, newly synthesized YY1 may differ from endogenous YY1 in compartmentalization or biological function. Only the newly synthesized exogenous YY1, not the endogenous YY1, bound with the high molecular weight Notch complex suppressed Notch transactivation. In this study, YY1 only partially suppressed the activation of CBF1-dependent luciferase activity transactivated by N1IC from 54- to 83-fold. Why did the exogenously expressed YY1 not completely block the activity of the reporter gene promoted by N1IC? Possibly, the amount of exogenous YY1 was insufficient to completely antagonize N1IC, and a very low concentration of N1IC can elicit its biological functions (45).

In the reporter gene assay, transfection of the transcription factor YY1-expressing plasmid alone did not activate the CBF1-response element (Fig. 5). This result agreed with the inability to precipitate YY1 with the 5′-biotinylated wild-type CBF1-response element in K562 cells by streptavidin-agarose beads (data not shown). However, YY1 could be precipitated with the wild-type CBF1-response element in Jurkat cells (Fig. 6*C*) and N1IC-expressing K562 cells. The luciferase reporter plasmid with the wild-type CBF1-response element could also be precipitated with anti-YY1 and anti-Notch1 C-terminal antibodies in the ChIP assay (Fig. 6*D*). Therefore, transcription factor YY1 regulated the transcriptional activity of the wild-type CBF1-response element via an association with the high molecular weight Notch complex containing CBF1.

The zinc finger domains, DNA binding domains of the transcription factor, are required for the association of YY1 with N1IC according to the GST fusion protein pull-down assay. Zinc finger domains are also important for the interaction of YY1 with several other cellular factors, including TBP, CBP/p300, TAFII55, TFIIB, E1A, c-Myc, SP1, and ATF/CREB (reviewed in Ref. 54). In mapping the binding region of the activated Notch1 receptor, the ANK domain of N1IC alone was associated with endogenous YY1 (Fig. 3*A*). The ANK domain has been detected in many proteins, such as cyclin-dependent kinase inhibitors, signal transduction and transcriptional regulators, cytoskeletal organizers, developmental regulators, and toxins (55, 56). This functional motif is a well known region for the interaction between proteins (38, 57). Although only the ANK domain of N1IC is sufficient to associate with YY1, it alone cannot transactivate CBF1-dependent Notch signaling (Fig. 1*B*).

The p300 protein associates with N1IC and acts as a transcriptional coactivator (28). The association between p300 and N1IC requires the CH3 region of p300, and deletion of the EP domain, a segment of 15 amino acid residues 3′ adjacent to the ANK domain, within N1IC destabilizes the interaction with p300 *in vivo* (28). The p300 protein also associates with YY1 through the C-terminal region of p300 that includes CH3 domain (46). The region of the four zinc fingers and the region

around residues 150–200 of YY1 are required for strong and weak association with p300, respectively (54). The association between N1IC and YY1 requires the ANK domain of N1IC and the four zinc fingers of YY1 (Fig. 3). Deletion of EP domain, ANKΔEP, did not affect the association of ANK domain of N1IC with YY1. This observation may exclude the possibility that N1IC associates with YY1 through p300 and explain the suppression effect of YY1 on CBF1-mediated transactivation activity of N1IC.

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