Evidence That C Promoter-binding Factor 1 Binding Is Required for Notch-1-mediated Repression of Activator Protein-1*

Received for publication, October 20, 2003, and in revised form, November 25, 2003
Published, JBC Papers in Press, November 26, 2003, DOI 10.1074/jbc.M311510200

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The Notch signaling pathway is a major system for controlling cell fate during the development of organisms as diverse as insects, nematodes, and mammals (1, 2). Although this pathway was originally identified and studied in Drosophila, genes homologous to components of the pathway have been cloned from numerous metazoan organisms. Vertebrates have four paralogs of Notch, Notch-1–4, and six genes encoding Notch ligands, Jagged-1 and -2, Delta-1, and Delta-like-1, -3, and -4 (3–6). The canonical Notch pathway involves activation of Notch through the binding of a transmembrane ligand on a neighboring cell. Ligand binding induces consecutive proteolytic cleavages catalyzed by metalloprotease tumor necrosis factor α-converting enzyme (7) and γ-secretase (8), which release the intracellular domain of Notch (NIC),† allowing it to undergo nuclear translocation. In the nucleus, NIC regulates gene transcription by binding to DNA-bound CSL (CBF1, Su(H), and Lag-1) proteins (9), thereby converting CSL from a repressor into an activator (10, 11). A host of coregulators, including mastermind (12, 13), CBP/p300 (14, 15), SMRT (16), and Ski-interacting protein (17), have been implicated in the control of NIC-mediated transactivation.

Given the diverse macromolecular interactions in which Notch engages and its crucial biological functions, not surprisingly, the domain organization of Notch is highly conserved (18). The Notch extracellular domain contains up to 36 tandem epidermal growth factor-like repeats and three cysteine rich Lin-12/Notch repeats, which function in ligand binding and Notch activation. The N terminus of NIC contains the RAM domain that physically associates with CSL proteins, which bind preferentially to the DNA sequence CGTGGGAA (9, 19) and variations thereof (19). The region C-terminal to the RAM domain, containing six cdc10/Ankyrin repeats, mediates additional protein interactions. The cdc10/Ankyrin repeats also interact weakly with CSL (20). The region C-terminal to the cdc10/Ankyrin domain has been implicated in various protein interactions and is important for transactivation (TAD) (21), although the PEST motif contributes to the control of Notch stability (22). Drosophila Notch and mammalian Notch-1, -2, and -3 contain nuclear localization signals (23) and an OPA sequence of unknown function (24).

We reported that the human Notch-1 intracellular domain (NIC-1) not only activates CSL-mediated transactivation but also represses AP-1-mediated transactivation (25). Because endogenous AP-1 target genes were repressed and similar concentrations of NIC-1 mediated activation versus repression, repression appeared to be physiologically relevant. Repression correlated with nuclear localization of NIC-1 and required the RAM domain. Repression was not accompanied by altered c-Jun N-terminal kinase-dependent signaling events, which activate AP-1. Based on the crucial roles of Notch in developmental processes such as hematopoiesis (26–30) and vasculogenesis (31–34) and the diverse regulatory functions of AP-1, including roles in hematopoiesis and vasculogenesis (35–38), Notch-AP-1 cross-talk is likely to be highly significant.

Since our discovery of Notch-AP-1 cross-talk, two additional reports described similar interactions (39, 40). NIC-1 repressed

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† The abbreviations used are: NIC, Notch intracellular domain; AP-1, activator protein-1; CBF1, C promoter-binding factor 1; MMP1, matrix metalloproteinase 1; RAM, RBP-Jκ-associated molecule; RT, reverse transcriptase; IMEM, Iscove’s modified Eagle’s medium; TPA, 12-O-tetradecanoylphorbol-13-acetate; HPRT, hypoxanthine phosphoribosyl transferase.
transcription of E6/E7 papillomavirus genes, and this was accompanied by up-regulation of Fra-1, a component of inhibitory AP-1 complexes, and down-regulation of c-Fos, a component of activating AP-1 complexes (39). However, Fra-1 and c-Fos (25) expression were unaffected by NIC-1 in K562 cells, under conditions in which AP-1-mediated transactivation was repressed. Furthermore, NIC-1 expression in K562 cells did not impact upon AP-1 DNA binding activity in nuclear extracts in vitro, indicating that NIC-1 did not induce the assembly of stable repressive heterodimers. Another member of the AP-1/ATF superfamily of basic leucine zipper transcription factors, B-ATF, is up-regulated by NIC-1 in Epstein-Barr virus-negative B-cell lymphoma BJAB cells (40). B-ATF is expressed highest in hematopoietic tissues and assembles into a repressive complex with c-Jun. Because B-ATF is not expressed in HeLa cells, NIC-1 represses AP-1 in these cells (25), and as noted above, NIC-1 does not affect AP-1 DNA binding activity in vitro, it is unlikely that B-ATF is a general mediator of inhibitory NIC-1-AP-1 cross-talk. Thus, although Fra-1 and B-ATF mediate NIC-1-AP-1 cross-talk in certain contexts, we expect that multiple modes of cross-talk exist, analogous to the inhibitory steroid receptor-AP-1 cross-talk, which has been studied for more than a decade without a unifying mechanism (41, 42).

Here, we investigated the mechanism of how Notch represses AP-1 and describe the identification of eight amino acids within the RAM domain that are crucial for CSL binding, activation, and repression. This analysis also revealed a powerful cell-sensing mechanism for selectively down-regulating NIC-1, which can be overridden by substituting only four amino acid residues with alanines.

MATERIALS AND METHODS

Plasmid Construction—The pNIC-1 expression vector encoding constitutively active Notch-1 (NIC-1) was described previously (25, 43–45). This vector was derived from the pBabe-puro retroviral vector (44) and includes a cDNA sequence encoding amino acids 1759–2056 of human Notch-1 with a Myc tag fused to its C terminus. Human NIC-1 deletion mutants were generated by the following methods. For NIC-1(Δ1779–1785), a cDNA sequence encoding amino acids 1759–2556 of human Notch-1 was subcloned into a pBluescript (pBS) vector with a mutation at the EcoO109I site (pBS/EcoO109) (39). This expression vector was then digested with SacII and EcoO109I. The resulting vector was ligated with the following oligonucleotide duplex: NIC-1(Δ1779–1785) top, TGAGGGCTTCAAGGCTGACCCCGCTC; NIC-1(Δ1779–1785) bottom, CGGCCGGTCTCCAGACACATTTGAGGCCC (the EcoO109I and EcoNI sites are in italics). For NIC-1(Δ1807–1813), pBabe/EcoO109) NIC-1 expression vector was digested with EcoO109 and EcoO109I, and the backbone vector was purified and ligated with the following oligonucleotide duplex: NIC-1(Δ1807–1813) top, CGCAACAGCTCCACGAGCCGCTCAGAGCTGCTCGTACTGAG; NIC-1(Δ1807–1813) bottom, GTCCTCGTCCCCACGAGGCGACCGTGGAA- GCCGCTTCCAGGCCTGAGCCGGTTCTC. The EcoO109I and EcoO109I sites are in italics. The deletion mutations were then subcloned into a pBabe-puro vector containing a C-terminal Myc epitope tag (EQKLISEEDL). For NIC-1(Δ1821–1827), NIC-1(Δ1827–1833), and NIC-1(Δ1834–1840), the NIC-1/BamHI-SacII fragment was subcloned into pBabe/SacII and then digested with EcoO109I and EcoO109I. The purified backbone of the vector was ligated with the NIC-1/EcoO109I/SacII) fragment containing Δ1821–1827, Δ1827–1833, or Δ1834–1840, which was generated by ligating two oligonucleotide duplexes. The NIC-1 constructs containing the deletion mutations were then subcloned into the pBabe-NIC-1/SacII-BamHI/Myc vector. NIC-1(Δ1759–1778) was generated by high fidelity PCR using a full-length NIC-1 expression vector as the template. NIC-1/BamHI-SacII containing Δ1759–1778 was generated by PCR using the following primer pairs: CCGGGATCCACCATGGGCGGATCCAG (the BamHI site is shown in italics, the initiator methionine is in bold type, and the NIC-1 sequences are underlined) and AGGGCGCGCCGACTTGAAGCTCATG (the SacII site is shown in italics, and the NIC-1 sequences are underlined). The PCR product was digested with BamHI and SacII. The pBabe-NIC-1 expression vector was digested with BamHI and SacII yielding pBabe-SacII-BamHI and NIC-1/BamHI-SacII. These fragments were purified, and NIC-1/BamHI-SacII was digested with SacII.

purified pBabe(SalI-BamHI) and NIC-1/SacII-SalI) fragments and the PCR product were ligated to generate NIC-1(Δ1759–1778).

Alanine substitution mutagenesis of NIC-1 was accomplished by insertion of oligonucleotide containing alanine substitutions into the NIC-1 expression vector after digestion with appropriate restriction enzymes. For NIC-1(Δ1759–1762A), NIC-1(Δ1763–1766A), and NIC-1(Δ1767–1770A), the pBabe-NIC-1 expression vector was digested with BamHI and SacII and then digested with another digestion with Sau3I. Purified pBabe(SalI-BamHI) and NIC-1(SacII-SalI) were ligated with oligonucleotides containing Ala substitutions. NIC-1(Δ1771–1774A) and NIC-1(Δ1775–1778A) were generated by sequential high fidelity PCR reactions. A short fragment from the NIC-1 BamHI site to the sequences that were substituted with Ala residues and a long fragment from the same sequences to the NIC-1 SacII site were generated by PCR using two primers in a strategy designed such that the two fragments had substantial overlapping sequences surrounding the Ala substitution region. The fragments were then used as templates to generate the NIC-1/BamHI-SacII fragment containing Ala substitutions using the forward primer for the short fragment and the reverse primer for the long fragment. After digestion with BamHI and SacII, this fragment was ligated with pBabe(SalI-BamHI) and NIC-1(SacII-SalI).

Cell Culture—The human erythroleukemia cell line K562 was maintained as described previously (25, 45). The cells were propagated in Iscove’s modified Eagle’s medium (Biofluids) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen) (complete IMEM) in a humidified incubator at 37 °C in the presence of 5% carbon dioxide.

Transient Transfections—K562 cells (5 × 10⁴) were collected by centrifugation at 240 × g for 5 min at 4 °C and resuspended in 4 ml of complete IMEM. Plasmid DNAs (1 μg of reporter and 2 μg of effector) were suspended in 150 μl of IMEM, incubated with Superfect (4 μl/μg DNA; Qiagen) for 10 min at room temperature, and then added to the cells. The cells were incubated for 28 h post-transfection and then treated with TPA (final concentration, 5 μM) or the vehicle (MeSO₄). After incubating for 16 h, the cells were harvested, and the cell lysates were assayed for luciferase activity. The luciferase activity was normalized by the protein content of the lysates, determined by Bradford assay using γ-globulin as a standard.

To determine the expression levels of wild-type NIC-1 and NIC-1 mutants, DNA was transiently transfected into K562 cells using DMRIE-C (Invitrogen). DNA (24 μg) and DMRIE-C reagent (72 μl) were diluted in 2.0 and 3.0 ml of OPTI-MEM I medium (Invitrogen), respectively. The two solutions were combined and incubated at room temperature for 45 min. K562 cells (9 × 10⁵) were collected by centrifugation at 240 × g for 5 min at 4 °C, washed once with ice-cold phosphate-buffered saline, and re-suspended in 1.0 ml of OPTI-MEM I medium. The cells were then added to the mixture of transfection components. After incubation at 37 °C in a CO₂ incubator for 4 h, complete IMEM (12 ml) containing 15% fetal bovine serum was added to the cells. The expression levels of wild-type NIC-1 and NIC-1 mutants were determined by Western blotting 40 h post-transfection.

Stable Transfection—K562 cells were stably transfected using LipofectAMINE 2000 (Invitrogen). K562 cells (1 × 10⁶) were collected by centrifugation at 240 × g for 5 min at 4 °C, washed once with ice-cold phosphate-buffered saline, and resuspended in 800 μl of OPTI-MEM I medium. Plasmid DNA (2 μg) was added to 100 μl of OPTI-MEM I medium, incubated with LipofectAMINE 2000 (4.5 μl/μg of DNA in 100 μl of OPTI-MEM I medium) for 30 min at room temperature, and then added to the cells. After incubating for 5 h at 37 °C in a 5% CO₂ incubator, complete IMEM (2 ml) containing 15% fetal bovine serum was added to the cells. Puromycin (1.5 μg/ml) (Sigma) was added 72 h post-transfection to select for stable transfectants, and expression levels were measured by Western blotting and by real time RT-PCR after 10–20 days of selection.

Western Blotting—To detect the expression of Myc-tagged NIC-1 and NIC-1 mutants, whole cell lysates were prepared in Nonidet P-40 lysis buffer (50 mM Heps, pH 7.4, 1.5 mM EDTA, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 20 μg/ml leupeptin). The lysates were cleared by centrifugation at 13,000 × g for 30 min at 4 °C. The supernatants were split into two aliquots and immunoprecipitated with either preimmune serum or anti-NIC-1 polyclonal antibodies. Immunoprecipitated complexes were adsorbed to protein A-Sepharose, and eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis on an 8% acrylamide gel. The proteins were transferred to an Immobilon P membrane (Millipore) and detected by immunoblotting with the anti-Myc tag polyclonal antibody (Upstate Biotechnology, Inc.; catalog number 06-5439). CBFI was detected by...
immunoblotting with anti-CBF1 polyclonal antisera raised against purified full-length CBF1.

Quantitative RT-PCR Analysis—Total RNA from K562 cells stably expressing the blank vector, wild-type NIC-1, or NIC-1 mutants was extracted with Trizol (Invitrogen). cDNA was synthesized by annealing RNA (2 μg) with 250 ng of a 1:4 mixture of random and oligo(dT) primers by heating at 68 °C for 10 min. After denaturation, the samples were incubated with Moloney murine leukemia virus reverse transcriptase (10 units/μl; Invitrogen) combined with 20 μM dithiothreitol, 1 mM dNTPs, and 2 units/μl RNasin (Promega) at 42 °C for 1 h. The reaction mixture was heat-inactivated at 95–100 °C for 5 min and diluted to a final volume of 200 μl. Quantitative real time RT-PCR reactions (25 μl) contained 2 μl of cDNA, 12.5 μl of SYBR Green (Applied Biosystems), and the appropriate primers. Product accumulation was monitored by SYBR Green fluorescence. The relative expression levels were determined from a standard curve of serial dilutions of cDNA samples. Forward and reverse primers for real time RT-PCR (5’-3’) were: human NIC-Myc, CCAGATCGCGCGCATC and GATATCGTGCTGCTCTCAGC; human hypoxanthine phosphoribosyl transferase, ATTGGTGGAGATGATCTCTAAACTTT and GCCAGTGCAAT-AGCTTCTGCTCCTCAGC; human Itch, AAACAGTCCCAAGTGGAAGCA-TATATCTTCCACAA; human CBF1, CGGACTTCG. MMP1, ACAAATCCCTTCTACCCGGAA and CCCTTTGAA-AAC-TCGCATCTGAG and CGTGACAGACGCATTATCAGTTG; and human hypoxanthine phosphoribosyl transferase, ATTGGTGGAGATGATCTCTCAACTTT and GCCAGTGCAAT-AGCTTCTGCTCCTCAGC.

RESULTS AND DISCUSSION

Structure/Function Analysis of NIC-1 RAM Domain Internal Deletion Mutants—Sequences within the highly conserved RAM domain (amino acids 1759–1849) of NIC-1 and amino acids 2105–2114 are crucial for CSL-mediated transactivation and AP-1 repression (25). To test whether distinct sequences mediate activation versus repression and whether CSL binding is required for repression, we systematically mutagenized the RAM domain and conducted a structure/function analysis. Seven RAM domain internal deletion mutants were generated, which together with NIC-1(Δ1784–1847), resulted in mutagenesis of the majority of RAM domain sequences (Fig. 1). Expression vectors encoding the mutants were transiently transfected into K562 erythroleukemia cells, and expression was assessed by immunoprecipitation of extracts with anti-NIC-1 antibody, followed by Western blotting with anti-Myc antibody. All of the mutants were expressed, and the expression levels did not differ greatly compared with wild-type NIC-1. However, NIC-1(ΔRAM) and NIC-1(Δ1759–1778) were reproducibly expressed at higher levels than wild-type NIC-1 and other deletion mutants (Fig. 2A).

To determine whether the mutants were competent to stably associate with endogenous CBF1, the blot of Fig. 2A was reprobed with anti-CBF1 antisera (Fig. 2B). Although CBF1 binding requires the RAM domain, the specific molecular determinants of binding have not been defined in detail. Endogenous CBF1 coimmunoprecipitated with wild-type NIC-1 and NIC-1(Δ1841–1847) but not the RAM domain deletion mutant NIC-1(ΔRAM). CBF1 coimmunoprecipitated with all RAM domain mutants except NIC-1(Δ1759–1778), which lacks the first 20 amino acids of the N terminus of the RAM domain. Similar to NIC-1(ΔRAM), no CBF1 was recovered upon immunoprecipitation of NIC-1(Δ1759–1778), whereas similar amounts of CBF1 were recovered upon immunoprecipitation of other RAM domain mutants and wild-type NIC-1.

The mutants were compared with wild-type NIC-1 for their capacity to activate transcription from a luciferase reporter plasmid containing four CBF1-binding sites (11). K562 cells were transiently transfected with the CBF1 reporter and a blank vector or expression vectors encoding wild-type or mutant NIC-1. Expression of wild-type NIC-1 strongly activated transcription of the CBF1 reporter, which binds endogenous CBF1 (Fig. 2C). Only the mutants that associate with CBF1 (Fig. 2B) activated the CBF1 luciferase reporter similar to wild-type NIC-1. NIC-1(ΔRAM) and NIC-1(Δ1759–1778), which do not stably associate with CBF1, were almost completely defective in activating the reporter.
transient transfection experiments (means ± S.E.). As expected, the expression levels of the alanine substitution mutants did not differ greatly, we assessed their capacity to stably associate with endogenous CBF1. The blot of Fig. 3A was reprobed with anti-CBF1 antisera. Three mutants (NIC-1(1759–1762A), NIC-1(1763–1766A), and NIC-1(1775–1778A)) associated with CBF1 similarly to wild-type NIC-1 (Fig. 3B). By contrast, the other two mutants, NIC-1(1767–1770A) and NIC-1(1771–1774A), did not coimmunoprecipitate with CBF1. Extensive development of the blot did not reveal any CBF1 immunoreactivity, indicating that these mutations completely abrogate stable complex formation. NIC-1(1767–1770A) and NIC-1(1771–1774A) lack WFP residues that were shown previously to be important for CSL binding in yeast two-hybrid and in vitro assays (9). In this previous study, mouse NIC-1 mutated in the RHG residues in the sequence RQHG (mM2–1) (amino acids 1752–1755) exhibited very weak CBF1 binding in a qualitative yeast two-hybrid assay, and a glutathione S-transferase fusion to mM2–1 bound trace amounts of in vitro translated [35S]methionine-labeled CBF1 (9). By contrast, our analysis of the binding of human NIC-1 mutants to endogenous CBF1 revealed that NIC-1(1763–1766A) associates with CBF1 similarly to wild-type NIC-1.

Because the expression levels of the alanine substitution mutants did not differ greatly, we assessed their capacity to induce CSL-mediated transactivation and to repress AP-1. The three mutants competent to bind CSL, NIC-1(1759–1762A), NIC-1(1763–1766A), and NIC-1(1775–1778A), activated transcription similarly to wild-type NIC-1 (Fig. 3C). As expected, the two mutants incapable of binding CSL, NIC-1(1767–1770A) and NIC-1(1771–1774A), were greatly impaired in their ability to activate transcription, similar to NIC-1(ΔRAM) and NIC-1(Δ1759–1778). Besides only weakly activating transcription, NIC-1(1767–1770A) and NIC-1(1771–1774A) only weakly re-
pressed AP-1 (Fig. 3D). These results provide evidence that amino acids 1767–1774 are crucial for binding CSL, for CSL-mediated transactivation, and for repressing AP-1.

Plotting the level of activation and repression obtained from each mutant yielded a linear relationship with $R^2 = 0.816$ (Fig. 4). Thus, CSL binding correlates with NIC-1-mediated repression of AP-1. Because the only known consequence of NIC-1 binding to CBF1 is to activate transcription of Notch target genes, these data are consistent with a model in which AP-1 repression requires activation of one or more target genes. The results with one mutant, NIC-1(Δ1841–1847), were not entirely consistent with this simple linear relationship. As noted in our previous study (25), NIC-1(Δ1841–1847) was partially compromised for activation but retained normal CBF1 binding and AP-1 repression. Thus, analogous to the 13 other mutants, the activity to repress AP-1 correlated with CBF1 binding. However, the efficiency of activation was modestly reduced upon deletion of amino acids 1841–1847. Linear regression analysis of activation versus repression data for all mutants except NIC-1(Δ1841–1847) yielded an $R^2$ value of 0.905.

A Powerful Cell-sensing Mechanism That Suppresses Protein Levels of NIC-1 and Transcriptionally Competent NIC-1 Mutants—Very low levels of NIC, below the threshold of detection by immunofluorescence, confer maximal CSL-mediated transactivation from reporter constructs in murine 3T3 cells (46). Based on this important finding, it is unclear whether only very low levels of NIC are generated upon Notch activation, whether cellular mechanisms suppress NIC levels, or whether cells repress transcription of ectopically expressed NIC. Wild-type NIC-1 and NIC-1 mutants are readily expressed upon transient transfection in K562 cells, and our previous work showed that stably transfected NIC-1 represses endogenous AP-1 target genes (25) and suppresses erythroid maturation of K562 cells (45). Studies were conducted to establish conditions to analyze the activity of the mutants studied in Figs. 2 and 3 in a chromosomal context in stably transfected K562 cells. Vectors encoding wild-type NIC-1, NIC-1 mutants, or an empty vector were transiently transfected into K562 cells, and stably transfected pools of cells were selected with puromycin. Expression of wild-type NIC-1 and NIC-1 mutants was assessed by immunoprecipitation with anti-NIC-1 antisera using extracts from pools of stably transfected cells. Immunoprecipitated proteins were detected by Western blotting with anti-Myc antibody. Even though wild-type NIC-1 and NIC-1 mutants were expressed at comparable levels when transiently transfected into K562 cells (Figs. 2A and 3A), wild-type NIC-1, NIC-1(1775–1778A), and NIC-1(Δ1841–1847), which retain transcriptional repression activities, were almost undetectable in the stably transfected cells. By contrast, mutants defective in both CSL-mediated transactivation and AP-1 repression (NIC-1 (ΔRAM), NIC-1(Δ1759–1778), NIC-1(1767–1770A), NIC-1(1771–1774A), and NIC-1(Δ2105–2114)) were highly expressed (Fig. 5, top (short exposure) and middle (long exposure) panels). α-Tubulin was expressed at similar levels in the extracts (Fig. 5, bottom panel), indicating that the differential expression levels of wild-type NIC-1 and NIC-1 mutants are not accompanied by overt changes in protein expression. Thus, the expression analysis yielded the surprising finding that transcriptionally compro-

Fig. 3. CBF1 binding, CSL-mediated transactivation, and AP-1 repressing activities of NIC-1 RAM domain alanine substitution mutants. A, Western blot analysis of wild-type NIC-1 and NIC-1 mutants. A blank vector or a NIC-1 expression vector was introduced into K562 cells by transient transfection. The cell lysates were immunoprecipitated with anti-NIC-1 antibody or preimmune sera (PI), and the bands were detected by Western blotting with anti-Myc antibody. The bracket identifies bands representing wild-type NIC-1 and mutants. B, Western blot analysis of endogenous CBF1 binding to NIC-1 and NIC-1 mutants. C, transient transfection analysis of CSL-mediated transactivation. D, transient transfection analysis of AP-1 repression. For C and D, K562 cells were transiently transfected with either CBF1 (C) or AP-1 (D) reporter vectors and the empty pBabe vector or vectors encoding NIC-1 or NIC-1 mutants. AP-1 reporter activity was induced by TPA treatment (5 nm, 16 h). The luciferase activity was normalized by the protein content of the lysate. The graph depicts data from three independent transient transfection experiments (means ± S.E.).
mised NIC-1 mutants accumulate at high levels, whereas expression of transcriptionally active NIC-1 mutants is suppressed. Because both classes of NIC-1 mutants are expressed similarly in transiently transfected K562 cells, this suggests that a time-dependent cell-sensing mechanism suppresses the levels of NIC-1 and transcriptionally competent NIC-1 mutants.

The results of Fig. 5 can be explained by one of the following two mechanisms. Transcriptionally competent NIC-1 levels cannot be sustained, and therefore NIC-1 is degraded. Alternatively, expression vectors encoding transcriptionally competent NIC-1 are selectively repressed upon integration into chromosomal DNA. To distinguish between these mechanisms, total RNA was isolated from the same stably transfected K562 cells used for the protein analysis of Fig. 5. The expression of exogenous NIC-1 mRNA transcripts was quantitated by real time PCR (Fig. 6). Primers were used that amplify the C terminal region of NIC-1 and the Myc tag to distinguish exogenous from endogenous NIC-1. The assays were conducted under linear conditions (Fig. 6A). Representative amplification curves for the detection of NIC-Myc and HPRT in K562 cells stably transfected with the empty vector (K562-Babe) or the NIC-1 expression vector (K562-NIC-1) are shown in Fig. 6B. Thermal dissociation of the PCR products revealed homogenous dissociation curves (Fig. 6C), indicative of a single major product for each primer set. Under conditions in which transcriptionally competent NIC-1 proteins were nearly undetectable (Fig. 5), the levels of RNA transcripts encoding NIC-1, transcriptionally competent NIC-1 mutants, and transcriptionally defective NIC-1 mutants were similar, differing by less than 2-fold. Thus, the very low protein expression levels of transcriptionally competent NIC-1 do not result from repression of the stably integrated expression vectors. Rather, a translational or post-translational mechanism suppresses the levels of active NIC-1 proteins in stably transfected K562 cells.

What mechanisms function translationally or post-translationally to strongly suppress the levels of transcriptionally competent NIC-1? NIC-1-dependent transcription might be tightly coupled to NIC-1 degradation, because this type of mechanism has been described for other transcriptional activators (47–49). Sequences conferring degradation via the ubiquitin-proteosome system have been localized within transcriptional activation domains (48). Transactivation domains can signal to the ubiquitination machinery, and intriguingly, activator ubiquitination appears to be required for transactivation in certain contexts (50). Alternatively, transcriptionally competent NIC-1 might activate a gene(s) that directly mediates NIC-1 degradation.

Because wild-type NIC-1 and NIC-1 mutants were expressed at similar levels in transiently transfected cells (Figs. 2 and 3), the latter mechanism seems to be the most likely, unless the coupling of activation and proteolysis requires a chromosomal template. SEL-10, an F-box protein of the cdc4 family, negatively regulates Notch signaling and catalyzes ubiquitination and degradation of NIC-1 (51, 52). However, SEL-10 interacts with the C-terminal portion of NIC-1, and the PEST domain present within this region is required for SEL-10-mediated degradation of NIC-1 (53). Because mutations that greatly modulate NIC-1 protein levels (Fig. 5) do not occur within the C terminus of NIC-1 or within the PEST domain (Fig. 1), it is unlikely that SEL-10 mediates the suppression of transcriptionally competent NIC-1 in stably transfected K562 cells.

Besides SEL-10, WW domain containing HECT domain ubiquitin ligases have been implicated in NIC-1 degradation. Dro sophila Suppressor of Deltex down-regulates Notch signaling (54, 55), whereas murine Itch binds and ubiquitinates NIC-1 in vitro (56). Although the molecular determinants of Itch binding
to NIC-1 are unknown, Itch binds a NIC-1 derivative containing the RAM domain and the ankyrin repeats but lacking the PEST domain.

To test whether NIC-1 induces expression of Itch and/or the highly homologous ubiquitin ligase Wwp1, we quantified the levels of Itch and Wwp1 mRNA transcripts in K562 cells stably expressing NIC-1 by real time RT-PCR. This analysis revealed that expression of endogenous Itch and Wwp1 mRNA was unaffected by NIC-1 (Fig. 7). However, because NIC-1 strongly repressed TPA-mediated induction of MMP1 expression (Fig. 7), NIC-1 was functional in the cells. These results indicate that the cell-sensing mechanism that suppresses the levels of transcriptionally competent NIC-1 in stably transfected K562 cells does not involve up-regulation of Itch or Wwp1 transcription. Our discovery of the differential expression of transcriptionally competent versus inactive NIC-1 derivatives in transiently versus stably transfected cells will provide a unique system for further elucidating mechanisms controlling Notch signaling via the regulation of NIC-1 protein levels.

In conclusion, we conducted a nonbiased, systematic analysis of the functionality of RAM domain sequences. Because the RAM domain of Notch shares no apparent sequence homology

**FIG. 6.** The inability of transcriptionally competent NIC-1 mutants to express at high levels in stably transfected K562 cells does not result from transcriptional repression of the integrated expression vectors. A, standard curves of SYBR green fluorescence signals obtained from dilutions of cDNA from K562 cells stably expressing NIC-1 (K562-NIC-1 cells) using a primer set specific for the C terminus of NIC-1 and the Myc-tag junction sequence (NIC-Myc) and HPRT. B, representative amplification plot of NIC-Myc and HPRT in K562-Babe and K562-NIC-1 cells. C, dissociation curve obtained from the amplicon shown in B (K562-NIC-1 cells). The single peak indicates the generation of one amplicon. D, real time RT-PCR analysis of NIC-Myc and HPRT RNA expression in K562 cells stably expressing wild-type NIC-1 or NIC-1 mutants.

**FIG. 7.** The inability of NIC-1 to express at high levels in stably transfected K562 cells does not result from the induction of Itch and Wwp1 HECT domain ubiquitin ligases. K562 cells were stably transfected with the empty vector pBabe or the NIC-1 expression vector pNIC-1. The relative levels of Itch, Wwp1, MMP1, and HPRT RNA transcripts in stably transfected pools of cells were quantitated by real time RT-PCR. Itch, Wwp1, and MMP1 transcript levels were normalized by the level of HPRT, which was constant under the various conditions. For the MMP1 analysis, the cells were treated with TPA (5 nM, 12 h) to induce MMP1 expression. Note that under conditions in which NIC-1 expression strongly reduces TPA-dependent MMP1 induction, Itch and Wwp1 transcript levels were not affected.
FIG. 8. A highly conserved subregion of the RAM domain containing amino acids essential for CBF1 binding, CSL-mediated transactivation, and AP-1 repression. Notch subtype sequences from various species were aligned. **Gray shading** denotes sequences from various species were aligned.

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to known proteins, despite being highly conserved among Notch proteins, and secondary structure predictions fail to reveal structural motifs, logical predictions cannot be made regarding RAM domain structure/function. Surprisingly, the majority of RAM domain sequences were not required for CBF1 binding, CSL-mediated transactivation, and AP-1 repression (Figs. 2 and 3). Only eight amino acids within the N terminus of the RAM domain were essential for CBF1 binding, CSL-mediated transactivation, and AP-1 repression. However, despite the high sequence conservation of amino acid residues at the extracellular N terminus of the RAM domain (Fig. 8), these residues had no apparent functional role. The correlation between CBF1 binding and AP-1 repression and the inability of the mutants to differentially affect CSL-mediated transactivation and AP-1 repression strongly suggest that CBF1 binding mediates AP-1 repression. Because the only activity ascribed to the NIC-1-CBF1 complex is to regulate Notch target genes, the data are consistent with a model in which AP-1 repression requires activation of one or more Notch target genes. However, mechanisms in which CBF1 binding is required but induction of Notch target genes is not cannot be unequivocally ruled out, even though there is no precedent for this type of mechanism. The studies also led to the unexpected finding that NIC-1 activity is tightly coupled to NIC-1 expression levels. Although the experiments involved modulation of NIC-1 activity via mutations, cellular mechanisms that up-regulate or down-regulate NIC-1 activity might be similarly coupled to the control of NIC-1 protein levels. Such a relationship has major implications for the control of Notch signaling and therefore diverse developmental processes.

Acknowledgments—We thank Dr. Anthony J. Capobianco for providing the initial NIC-1 constructs and Dr. Diane Hayward for providing all other materials. We thank Kirby Johnson and Soumen Paul for critical reviews of the manuscript.

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