Repression of Activator Protein-1-mediated Transcriptional Activation by the Notch-1 Intracellular Domain*

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Developmental decisions that control cell fate are commonly regulated by the Notch signaling pathway. Activation of transmembrane Notch receptors results in proteolytic liberation of the intracellular domain of Notch, which translocates into the nucleus, binds a repressor (C promoter binding factor I/RBP-Jκ, Su(H), and Lag-1 (CSL)), and induces target genes. We found that the intracellular domain of human Notch-1 (NIC-1) represses activator protein-1 (AP-1)-mediated transactivation. Because numerous genes that control immune and inflammatory responses are AP-1-dependent and Notch regulates immune cell function, we investigated the underlying molecular mechanisms. Repression of AP-1 by NIC-1 did not represent a general inhibitory effect on transcription because nuclear factor κB-dependent transcription and transcription driven by a constitutive promoter and enhancer were not affected by NIC-1. The physiological relevance of the repression was supported by the facts that repression was apparent in multiple cell lines, endogenous AP-1 target genes were repressed, and similar concentrations of NIC-1 were required for CSL-dependent activation and AP-1 repression. The RBP-Jκ-associated molecule domain of NIC-1 that mediates CSL binding and distinct sequences necessary for transactivation were required for repression. However, there was not a strict correlation between the sequence requirements for CSL-dependent activation and AP-1 repression. Repression correlated with predominant nuclear localization of NIC-1 and was not accompanied by disruption of c-Jun amino-terminal kinase-dependent signaling events required for AP-1 activation or by defective AP-1 DNA binding activity. These results provide evidence for negative cross-talk between Notch and AP-1, which may have important consequences for controlling diverse biological processes.

The highly conserved Notch signaling pathway controls cell fate decisions in organisms as diverse as insects, nematodes, and mammals (1–3). Examples of biological processes regulated by Notch signaling include neurogenesis (4, 5), hematopoiesis (6), vasculogenesis (7), and cortical neurite growth (5). Four paralogs of Notch, Notch 1–4, and five Notch ligand genes, Jagged-1, Jagged-2, Delta-1, Delta-like 1, and Delta-like 3, have been identified in vertebrates (8). Two modes of Notch signaling have been proposed, involving either interaction of the intracellular domain of Notch (NIC)† with CBF1/RBP-Jκ, Su(H), and Lag-1 (CSL) repressor proteins (9–11) or a CSL-independent mechanism (12, 13). In the canonical CSL-dependent mechanism, activation of a Notch transmembrane receptor by a transmembrane ligand on a neighboring cell results in two consecutive proteolytic cleavages, allowing for release and nuclear translocation of NIC (14–16). Nuclear NIC physically interacts with CSL bound with sequence specificity to promoters of target genes (11). Additional components such as mastermind (17, 18) and ski-interacting protein (19) assemble into the NIC-CSL nucleoprotein complex and are important for transactivation. CSL-independent signaling apparently also involves transcriptional regulation (12), but there is still much to be learned about the requisite components and the underlying mechanisms.

Because Notch has multiple conserved domains with the potential to be protein docking sites, Notch might act as a scaffold to assemble complexes containing components of the Notch and other signaling pathways. As with any complex signaling system, physiological functions mediated by Notch are likely to depend on how Notch signals integrate with signals emanating from other pathways. Indeed, Notch signaling interacts with multiple signaling pathways including Ras (13, 20–23), Wnt (24–26), T-cell receptor (27), granulocyte colony-stimulating factor (28), granulocyte macrophage colony-stimulating factor (28), and NF-κB (29–32).

Multiple lines of evidence support the existence of physiological cross-talk between the Notch and Ras pathways. Notch mutants in Drosophila have elevated levels of the Ras-regulated stress-activated kinase JNK (13), suggesting negative cross-talk between Notch and JNK pathways. In addition,
Notch-1 and Notch-2 inhibit the E47 transcription factor, and this involves inhibition of Ras signaling, which is required for E47 activity (21). Moreover, during vulval development in Cae- norhabditis elegans, Notch-mediated transcriptional activation of the MAPK phosphatase LIP-1, which counteracts Ras-dependent MAPK signaling, establishes the basis for opposing Notch and Ras signals (23). In contrast, Ras signals are required for anchorage-independent growth of cancer cell lines derived from Notch-4-expressing transgenic mice (22). Although the consequences of interactions between Notch and Ras are just beginning to be investigated, such interactions would likely affect the activity of the transcription factor AP-1, a major nuclear target of Ras.

AP-1 consists of homodimers of Jun family members or heterodimers of Jun and Fos proteins (33). Growth factors, cytokines, and tumor promoters activate AP-1 as an integral step in their mechanism of action (34), establishing a crucial role for AP-1 in many cellular processes including proliferation, differentiation, and survival. Dysregulation of AP-1 is a prototypical mechanism of tumor promotion (35). Disruption of Notch signaling can also transform cells (36, 37) and has been hypothesized to cause leukemogenesis (reviewed in Refs. 38 and 39).

The mechanism of Ras-dependent AP-1 activation involves phosphorylation of c-Jun and Jun family members on amino-terminal serines (serines 63 and 73 for c-Jun) (40). These modifications are often mediated by JNK (41), but p38 can also catalyze phosphorylation at these sites (42). Phosphorylation of threonine 231 and serine 249 near the DNA binding domain of c-Jun represses DNA binding, and dephosphorylation confers high-affinity binding (43). c-Jun phosphorylated at serines 63 and 73 interacts with the coactivator CBP/p300 (44). CBP/p300 confers transcriptional activation via histone and nonhistone protein acetylation (45, 46), although the mechanism by which AP-1 utilizes CBP/p300 is unclear. An additional AP-1 coactivator is Jab1 (47), a component of the COP9 signalosome complex (48), which stabilizes DNA-bound AP-1 complexes (47). The AP-1 stimulatory activity of Jab1 has been reported to be JNK-dependent (49) and -independent (50) in different systems. Thus, AP-1 is a dynamically regulated nuclear effector of Ras and integrates diverse cellular signals. Here, we show that the intracellular domain of human Notch-1 (NIC-1) strongly represses AP-1-mediated transactivation. Given the growing array of biological processes that Notch controls, cross-talk between Notch and AP-1 is likely to have important physiological and pathophysiological implications.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The pBabe-NIC-1 (pNIC-1) expression vector encoding constitutively active Notch-1 (NIC-1) was described previously (36, 37, 51). This vector was derived from the pBabe-puro retroviral vector and includes a cDNA sequence encoding amino acids 1759–2556 of human Notch-1 with a Myc tag fused to its carboxyl terminus. Human NIC-1 deletion mutants were generated by PCR using a full-length human Notch-1 expression vector as the template. The Notch-dependent reporter plasmid containing four CBP1 binding sites and a simian virus 40 promoter fused to luciferase (p4xCBF1Luc) was described previously (52) (a gift of Dr. Diane Hayward, Johns Hopkins Medical School). The AP-1 reporter plasmid (p1xAP1Luc) containing a collagenase promoter fragment (–73/+67) with a single AP-1 binding site in the luciferase reporter vector pGL2-basic (Promega) was described previously (53) (a gift of Dr. Nancy Colburn, National Cancer Institute). The NFkB reporter plasmid containing four NFkB binding sites and a minimal promoter fused to luciferase was a kind gift of Dr. Shigeki Miyamoto (University of Wisconsin Medical School). The pBabe-H-Ras(12V) expression vector encoding constitutively active H-Ras was a kind gift of Dr. Channing Der (University of North Carolina-Chapel Hill). This vector was derived from the pBabe-puro retroviral vector and includes a cDNA encoding H-Ras with a Gly to Val mutation at amino acid 12.

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

**Indirect Immunofluorescence**—HeLa cells (2.5 × 10^6) were seeded into 6-well plates and transfected with 2 μg of the indicated NIC-1 construct. Cells were transfected using 5 μl of LipofectAMINE in a total volume of 20 μl of OptiMEM; 24 h after transfection, cells were plated at 5.0 × 10^4 cells/well on 4-chamber glass slides. Indirect immunofluorescence was performed as described previously (37) using bTAN15A (54) for the primary antibody, followed by incubation with a donkey anti-rat Cy3-conjugated secondary antibody. Proteins were photographed on a Zeiss Axiophot fluorescence microscope with a Hamamatsu digital camera at ×400 magnification.

**Stable Transfection**—K562 cells were stably transfected by electroporation with a Bio-Rad Gene pulser electroporator. Cells (5 × 10^6) were washed with ice-cold PBS, resuspended in 0.5 ml of ice-cold PBS, mixed with 5 μg of linearized plasmid DNA, and subjected to electroporation (960 microfarads; 220 V) in a 0.4-cm-wide electroporation cuvette (BTX). pBabe and pNIC-1 were linearized with NotI. Cells were transfected with 20 μl of complete IMEM, grown for 48 h, and diluted in complete IMEM containing 1.5 μg/ml puromycin (pools of K562-Babe and K562-NIC-1 cells). Stably transfected cells were analyzed for erythroid differentiation by benzidine staining as soon as the pools were generated to reduce the probability of phenotypic changes that may result from prolonged growth.

**Retroviral Infection**—Modified 293 human embryonic kidney cells were grown in 10-cm dishes until they were subconfluent, and then they were cotransfected with plasmid DNA (15 μg) and pMD.G (6 μg) by the calcium phosphate transfection method as described previously (55). The medium was changed once after 10 h of transfection to remove the calcium phosphate. The pMD.G expression vector encodes the viral envelope protein vesicular stomatitis virus G protein. The modified 293 cells were previously stably transfected with pol and gag genes (gift of Shigeki Miyamoto, University of Wisconsin Medical School). After incubation for an additional 12 h, the medium was removed, and K562 cells (10 ml; 3 × 10^6 cells/ml) were added with polybrene (4 μg/ml) in complete IMEM and incubated for 36 h. The infected cells were separated from adherent 293 cells and then subjected to immunoprecipitation analysis.

**Stable Transfections**—K562 cells (5 × 10^6) were collected by centrifugation at 240 × g for 8 min at 4 °C and resuspended in 4 ml of complete IMEM. Plasmid DNAs (1 μg of reporter and 2 μg of effector) were added to 150 μl of IMEM, incubated with Superfect (4 μl/μg DNA; Qiagen) for 10 min at room temperature, and then added to cells. For transient transfection of HeLa cells, cells (2 × 10^5) were seeded in a 6-well plate 1 day before transfection. On the day of transfection, medium was removed, cells were washed once with ice-cold PBS, and 600 μl of complete DMEM was added. Plasmid DNAs (1 μg of reporter and 2 μg of effector) were added to 150 μl of DMEM, incubated with 12 μl of Superfect (Qiagen) for 10 min at room temperature, and then added to cells. After a 3-h incubation, the mixture was removed, cells were washed once with ice-cold PBS, and 4 ml of fresh complete DMEM was added.

For each transfection, cells were incubated for 26 h after transfection and then treated with TPA (final concentration, 5 nM) or the vehicle (Me2SO). After incubation for another 12 or 16 h, cells were harvested, and assayed for luciferase activity. Specific activity32P-labeled probes generated by random priming cDNA were generated to reduce the probability of phenotypic changes that may result from prolonged growth.

**Western Blotting**—To detect the expression of Myc-tagged wild-type NIC-1 and NIC-1 mutants, whole cell lysates were prepared in Nonidet

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**Note:** The above text is a natural language description of the experimental procedures and results presented in the original document. The text has been reformatted for clarity and readability while maintaining the original content's meaning and context. For more detailed information, including the methods and results, please refer to the original source.
Repression of AP-1 by Notch

RESULTS AND DISCUSSION

Repression of Endogenous AP-1 by the Notch-1 Intracellular Domain—We showed previously that NIC-1 represses transcriptional activation of IL-8 upon erythroid maturation of K562 erythroleukemia cells (51). These cells express endogenous Notch-1 and are competent to carry out strong CSL-dependent transcriptional activation (51). To investigate mechanisms underlying the repression, we tested whether NIC-1 antagonizes factors required for induction of IL-8 transcription. AP-1 (58) and NF-κB binding sites (59) on the IL-8 promoter are critical for transcriptional activation of IL-8 in response to diverse signals, although the relative importance of the two sites differs in different cell systems (60, 61).

AP-1 and NF-κB-responsive luciferase reporter constructs and a NIC-1 expression vector were transiently cotransfected into K562 cells. Previously, we showed that this NIC-1 expression vector confers low level expression of NIC-1 protein in K562 cells (51) and other cell types (36, 37). NIC-1 expression in K562 cells strongly activated transcription of a luciferase reporter that binds endogenous CSL proteins (Fig. 1A). Treatment of cells with the phorbol ester TPA to activate endogenous AP-1 strongly induced the activity of an AP-1 reporter containing a collagenase 1 (MMP1) promoter with a single AP-1 site (62). Under identical conditions in which the CSL reporter was activated by NIC-1, NIC-1 repressed AP-1 reporter activity (Fig. 1B). We asked whether repression was dependent upon the context of the AP-1 binding site within the promoter of the reporter. The degree of repression seen with a distinct AP-1 reporter containing tandem AP-1 binding sites upstream of a β-globin promoter (63) (Fig. 1C) was comparable with that seen with the MMP1 promoter (Fig. 1B), suggesting that repression is not context-dependent. Although the repression was strong with both AP-1 reporters, a component of the TPA-induced AP-1 reporter activity (~30%) was insensitive to NIC-1.

Because IL-8 transcription is also controlled via an NF-κB binding site on the IL-8 promoter (58, 59), we asked whether NIC-1 affects NF-κB-dependent transcription. Previous studies in different systems showed that NIC-1 can repress (29, 30) and activate NF-κB (31). Upon transient transfection into K562 cells, an NF-κB reporter gene containing three NF-κB binding sites was strongly activated by treatment of cells with TPA (Fig. 1D). In contrast to the AP-1 reporters, NIC-1 had no effect on NF-κB-dependent reporter activity (Fig. 1D). To further assess the specificity of the NIC-1-mediated AP-1 repression, we asked whether NIC-1 influenced the activity of a constitutively active promoter, the human α-globin promoter (pGL3α-gal) (Fig. 1E), and a constitutively active enhancer, the CMV enhancer (pCMVβgal) (Fig. 1F). TPA treatment increased the activity of pGL3α-gal by ~3-fold and strongly increased the activity of pCMVβgal. NIC-1 increased the basal activity of pGL3α-gal by ~80% without affecting the TPA-induced activity. NIC-1 had no effect on the basal activity of pCMVβgal but increased the TPA-induced activity by ~2-fold. Thus, NIC-1 represses AP-1-dependent transactivation in a context-independent manner in transient transfection assays. The lack of repression of NF-κB-dependent transcription and pGL3α-gal and pCMVβgal suggests that there is a considerable degree of specificity for the repression. These results are inconsistent with models in which NIC-1 has a general repressive effect on components of the basal transcription machinery or on all forms of activated transcription.

The failure of NIC-1 to completely repress AP-1-mediated transactivation could be due to an intrinsically resistant component of AP-1 activity or the inability of NIC-1 to overcome the strong stimulation of AP-1 activity achieved with a maximally effective TPA concentration. To distinguish between these pos-
sibilities, K562 cells were treated with a range of TPA concentrations, and the degree of inhibition by NIC-1 was compared under conditions of submaximal and maximal stimulation (Fig. 2). At all TPA concentrations, a resistant component of activity was apparent, and the degree of inhibition was not higher upon submaximal stimulation of AP-1. These results show that NIC-1 inhibits /H1101170% of the TPA-inducible AP-1 activity, whereas a second component of the AP-1 activity is resistant to repression by NIC-1.

To determine whether AP-1 activity induced by a distinct stimulus was inhibited by NIC-1 and whether a component of the activity was resistant to NIC-1, we activated endogenous AP-1 by transient expression of constitutively active H-Ras(12V). H-Ras(12V) activated AP-1 reporter activity, and NIC-1 almost completely inhibited H-Ras(12V)-activated AP-1 (Fig. 3). H-Ras(12V) expression slightly activated CSL-dependent reporter activity in the absence of exogenous NIC-1 and did not significantly influence NIC-1-dependent activation of the CSL reporter. Thus, activation of AP-1 by TPA or H-Ras(12V) was strongly inhibited by NIC-1. However, the NIC-1-resistant component of AP-1 activity (Figs. 1 and 2) was only apparent when TPA was used to activate AP-1.

**Is Repression of AP-1-mediated Transactivation by NIC-1 Physiologically Relevant?**—As described under “Introduction,” several reports have provided evidence for functional cross-talk between Notch and Ras pathways, establishing a strong precedent for physiological Notch-Ras interactions. Activation of JNK and p38, downstream of Ras, leads to phosphorylation of serines 63 and 73 on the amino terminus of c-Jun (and conserved sites of other Jun family members), thereby stimulating AP-1-mediated transcription. Our discovery that NIC-1 represses AP-1-mediated transcription may reflect a previously unrecognized component of Notch-Ras cross-talk. We reasoned that if the repression of AP-1-mediated transactivation by NIC-1 is physiological, then repression would not be unique to K562 cells, endogenous AP-1 target genes would be repressed, and repression would not require higher concentrations of NIC-1 than required for activation of CSL-dependent transcription. These issues were addressed in the following experiments.

To assess whether repression of AP-1-mediated transactivation by NIC-1 was unique to K562 cells, we asked whether NIC-1 represses endogenous AP-1 in HeLa cells (Fig. 4). NIC-1 strongly activated CSL-dependent reporter activity in HeLa cells. AP-1 reporter activity was strongly induced upon treatment of HeLa cells with TPA. Similar to K562 cells (Figs. 1 and 2), repression of AP-1 by NIC-1 was observed in HeLa cells, indicating that repression is a general feature of CSL-dependent transcription.
Repression of AP-1 by Notch

As mentioned above, induction of endogenous IL-8 expression upon erythroid maturation of K562 cells was repressed by stably expressed NIC-1 (51). To define whether NIC-1 derepresses endogenous AP-1 target genes (64, 65) in a context that is not confounded by the complexities of cellular differentiation, IL-8 and MMP1 were activated by treatment of K562-Babe and K562-NIC-1 cells with TPA, and steady-state mRNA levels were measured by Northern blotting. Maximal induction of IL-8 by TPA requires both AP-1 and NF-kB binding sites, and the relative importance of the sites varies in different systems (58, 59). MMP1 is a prototypical AP-1 target gene, although Ets factors can activate MMP1 via synergism with AP-1 (66) or repress (67) MMP1. TPA treatment strongly induced IL-8 and MMP1 transcript levels in K562-Babe cells with a stably transfected empty vector, whereas induction was considerably lower in K562-NIC-1 cells containing stably transfected NIC-1 (Fig. 5). The degree of repression of endogenous IL-8 and MMP1 transcription was similar to that seen in the transient transfection assays of Figs. 1 and 2. Because the AP-1-responsive p1xAP1luc reporter of Fig. 1 and the IL-8 promoter lack Ets sites, NIC-1 does not require coupled AP-1 and Ets sites to confer repression.

To investigate the specificity of the repression in a chromosomal context, we measured the levels of IkBa transcripts after TPA treatment of K562-Babe and K562-NIC-1 cells. IkBa is a prototypical NF-kB target gene (68), and TPA activates IkBa transcription via a mechanism involving NF-kB activation. NIC-1 had no effect on TPA induction of IkBa transcripts, consistent with the experiment of Fig. 1D showing no effect of NIC-1 on TPA induction of an NF-kB reporter in transient transfection assays. The failure of NIC-1 to influence NF-kB-driven transcription in K562 cells suggests that previous reports of NIC-1-mediated repression (29,30) and activation of NF-kB-dependent transcription (31) reflect cell type-specific actions. Our results show that NIC-1 represses the endogenous AP-1 target genes IL-8 and MMP1, and the lack of effect of NIC-1 on induction of IkBa confirms the specificity of the response.

If repression of AP-1-mediated transactivation by NIC-1 is physiologically relevant, then repression should occur at NIC-1 concentrations resembling that required to activate CSL-dependent transcription. On the other hand, if repression requires considerably higher concentrations of NIC-1, this would be inconsistent with a physiological mechanism. To address this issue, we compared the concentrations of NIC-1 expression vector required for CSL-dependent activation and AP-1 repression (Fig. 6). Transfection of K562 cells with increasing amounts of NIC-1 expression vector while maintaining a constant total DNA concentration induced a concentration-dependent activation of CSL-dependent reporter activity. Similarly, increasing amounts of NIC-1 expression vector decreased AP-1-dependent reporter activity as a function of vector concentration. The concentration-response curves for CSL-dependent activation and AP-1 repression were similar. However, the curve for AP-1 repression was slightly shifted to the left, showing that slightly less NIC-1 expression vector was required to achieve a comparable degree of AP-1 repression versus CSL-dependent activation. Thus, at NIC-1 concentrations capable of conferring CSL-dependent activation, the well-established physiological action of NIC-1, NIC-1 represses AP-1, providing strong evidence that AP-1 repression would occur under physiological conditions. Taken together with the facts that repression occurs in multiple cell types and that endogenous AP-1 target genes are repressed, it is likely that NIC-1 engages in physiological cross-talk with the AP-1 pathway. It is therefore of intrinsic interest to elucidate the molecular mechanisms underlying the cross-talk.



2), NIC-1 repressed AP-1-mediated activation, with a component of the activity being resistant to NIC-1. Thus, the repression of AP-1-mediated activation by NIC-1 is not unique to K562 cells, suggesting that repression would be apparent in diverse systems. Because AP-1 controls the expression of a plethora of genes mediating immune and inflammatory responses and NIC-1 has important activities to control immune cell function, cross-talk between Notch and AP-1 pathways would likely have important biological consequences.

As mentioned above, induction of endogenous IL-8 expression upon erythroid maturation of K562 cells was repressed by stably expressed NIC-1 (51). To define whether NIC-1 derepresses endogenous AP-1 target genes (64, 65) in a context that is not confounded by the complexities of cellular differentiation, IL-8 and MMP1 were activated by treatment of K562-Babe and K562-NIC-1 cells with TPA, and steady-state mRNA levels were measured by Northern blotting. Maximal induction of IL-8 by TPA requires both AP-1 and NF-kB binding sites, and

![Graph](image-url)

**Fig. 3.** NIC-1 completely inhibits H-Ras(12V)-inducible AP-1 activity. K562 cells were transiently cotransfected with pBabe, pNIC-1, or pBabe-H-Ras(12V) and either the p1xAP1luc or p4xCBF1luc reporter vectors. Luciferase activity was normalized by the protein content of the lysate. The graph depicts averaged data from three independent transient transfection experiments (mean ± S.E.).

![Graph](image-url)

**Fig. 4.** NIC-1 represses AP-1-mediated transactivation in HeLa cells. HeLa cells were transiently cotransfected with pBabe or pNIC-1 and reporter vectors containing four CBF1 (p4xCBF1luc) binding sites or one AP-1 (p1xAP1luc) binding site. AP-1 reporter activity was induced by TPA treatment (5 ng, 16 h). The luciferase activity was normalized by the protein content of the lysate. The graph depicts averaged data from three independent transient transfection experiments (mean ± S.E.).
and NIC-1 mutants was assessed by immunoprecipitation with an anti-NIC-1 antibody with extracts isolated from transfected K562 cells, and immunoprecipitated proteins were detected by Western blotting with an anti-myc antibody. All mutants were expressed, and the expression levels did not differ greatly (Fig. 7B). The blot was also probed with anti-CBF1 antisera to assess the recovery of CBF1 in the immunoprecipitates (Fig. 7C). CBF1 coimmunoprecipitated with wild-type NIC-1, NIC-1-(1842–1848), and NIC-1-(2105–2114). In contrast, almost no CBF1 was recovered upon immunoprecipitation of NIC-1-(1848–2556) and NIC-1-(1820–2556), which lack the entire RAM domain and a major portion of the RAM domain, respectively.

The mutants were compared with wild-type NIC-1 for their ability to activate CSL-dependent transcription and to repress AP-1. As expected, NIC-1-(1848–2556) only weakly induced CSL reporter activity. Surprisingly, NIC-1-(1848–2556) did not repress AP-1 reporter activity (Fig. 7D). NIC-1-(1820–2556) had a similar behavior, only weakly activating CSL-dependent transcription and weakly repressing AP-1. Thus, analysis of constructs with complete and partial RAM domain deletions revealed a critical requirement of RAM domain sequences for CSL-dependent activation and AP-1 repression. NIC-1-(1842–1848) conferred less CSL-dependent activation than did wild-type NIC-1, whereas it repressed AP-1 slightly better than did wild-type NIC-1. Intriguingly, amino acids 1842–1848 are selectively required for maximal CSL-dependent activation but not for repression. An additional mutant, NIC-1-(Δ2105–2114), known to be strongly impaired in conferring transactivation (37), was also tested. As expected, NIC-1-(Δ2105–2114)
weakly activated CSL-dependent reporter activity, similar to NIC-1-(1848–2556) and NIC-1-(1820–2556); NIC-1-(H9004–2105–2114) did not repress AP-1 reporter activity. Because CBF1 coimmunoprecipitated with NIC-1-(H9004–2105–2114) and NIC-1-(H9004–2105–2114) was not competent for AP-1 repression, clearly CBF1 binding is insufficient for AP-1 repression. These results provide evidence that sequences within the highly conserved RAM domain of NIC-1 and amino acids 2105–2114 are critical for CSL-dependent activation and AP-1 repression. Despite these common sequence requirements for CSL-dependent activation and AP-1 repression, the behavior of NIC-1-(H9004–1842–1848) is consistent with distinct but overlapping sequence requirements within the RAM domain. The RAM domain requirement for AP-1 repression constitutes a previously undescribed activity of this evolutionarily conserved domain (Fig. 7E); the RAM domain was only known to mediate CSL binding and CSL-dependent activation.

As noted above, NIC has been shown to inhibit H-Ras-mediated activation of E47-dependent transactivation in transient transfection assays (21). In that study, it was also shown in transient assays in 3T3 cells that NIC-2 inhibited transactivation mediated by the GAL4 DNA binding domain fused to a portion of c-Jun and that inhibition did not require the RAM domain. This contrasts with our results in which the intact RAM domain (amino acids 1759–1847) and a portion of the RAM domain (amino acids 1759–1819) were absolutely required for repression of endogenous AP-1. This difference may reflect cell type-specific differences in the behavior of NIC, different influences of NIC on GAL4-c-Jun and endogenous AP-1, or differences between the activities of NIC-1 and NIC-2. We assessed the impact of NIC-1 on transactivation mediated by GAL4 fused to the c-Jun activation domain (GAL4-c-Jun) in transient assays in K562 cells. NIC-1 did not significantly inhibit GAL4-c-Jun-mediated transactivation.3

Given that NIC-1 localizes predominantly to the nucleus, we reasoned that repression might occur within the nucleus. However, AP-1 is known to be activated via phosphorylation of amino-terminal serines of c-Jun and Jun family members, and therefore it is conceivable that NIC-1 disrupts membrane or cytoplasmic signaling events required for AP-1 phosphorylation and subsequent activation. Importantly, the experiments of Figs. 1 and 5 used TPA to activate NF-κB-dependent transcription, and NIC-1 had no effect on the TPA-dependent induction. This suggests that if NIC-1 inhibits TPA-dependent signaling events, these events would not be shared by the NF-κB and AP-1 activation pathways.

To define whether repression requires nuclear localization of NIC-1, NIC-1 derivatives were tested in which NES or NLS sequences were engineered at the carboxyl terminus (Fig. 8A). It was shown previously by indirect immunofluorescence assays that NIC-1/NLS, like NIC-1, has a predominant nuclear localization, whereas NIC-1/NES localizes to the cytoplasm and to the nucleus (37). Given the established function of NES

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3 J. Chu and E. H. Bresnick, unpublished data.
sequences (71), it is likely that the cytoplasmic and nuclear distribution of NIC-1/NES reflects active shuttling of NIC-1/NES between the two cell compartments. We examined the subcellular localization of these NIC-1 derivatives in HeLa cells and tested their ability to activate CSL-dependent transcription and repress AP-1. The subcellular localization of the constructs (Fig. 8B) was similar to that described previously (37). Whereas NIC-1/NLS resembled NIC-1 in activating CSL-dependent reporter activity and weakly repressed AP-1 reporter activity (Fig. 8C). These results provide a correlation between predominant nuclear localization and strong repression of AP-1, supporting a model in which repression occurs within the nucleus.

Does NIC-1 Inhibit Signaling Events Required for AP-1 Activation?—If repression of AP-1 by NIC-1 occurs within the nucleus, this would be inconsistent with an inhibitory effect of NIC-1 on membrane and cytoplasmic signaling events necessary for AP-1 activation. To define the influence of NIC-1 on such signaling events, we measured the phosphorylation state of relevant signaling components by Western blot analysis with phospho-specific antibodies. An inhibitory effect of NIC-1 on signaling would be manifested by disrupted signaling downstream of the inhibited step and normal signaling upstream of the inhibited step. Multiple MAPKs have been reported to be activated by TPA including JNK, p38, and ERK1/2. Analysis of the phosphorylation state of these MAPK subtypes, under identical growth conditions as the transient transfection and Northern analyses, revealed that TPA induced phosphorylation of these components to varying degrees but had no measurable effect on the expression levels of the components (Fig. 9A). Stably transfected NIC-1 did not affect TPA-induced or basal phosphorylation of JNK or p38; basal phosphorylation and TPA-induced ERK1/2 phosphorylation were slightly higher in K562-NIC-1 cells than in K562-Babe cells. Because the identical stably transfected cells that were subjected to Western blot analysis were analyzed by Northern blotting for induction of IL-8 and MMP1 expression and these genes were repressed by...
NIC-1 (Fig. 5), it is unlikely that impaired phosphorylation of MAPks is the mechanism underlying AP-1 repression.

Activation of MAPks can result in nuclear translocation of the activated enzymes (72). As noted above, one consequence of JNK and p38 activation is phosphorylation of serines 63 and 73 of c-Jun and conserved serines of Jun family members. We tested whether Jun phosphorylation was impaired in K562-NIC-1 cells using antibodies specific for phosphorylated serine 73 of c-Jun and the corresponding site of JunD and phosphorylated serine 63 of c-Jun. NIC-1 did not affect c-Jun protein levels, nor did it influence phosphorylation at either site (serine 73, Fig. 9A; serine 63, data not shown). Thus, it is unlikely that altered synthesis or disrupted phosphorylation of Jun proteins causes decreased AP-1 activity. The lack of effect of NIC-1 on serine 63 and 73 phosphorylation is consistent with the failure of NIC-1 to inhibit JNK and p38 phosphorylation; inhibition of JNK and p38 phosphorylation should decrease phosphorylation of serines 63 and 73 of c-Jun and the corresponding sites of JunD. Furthermore, if NIC-1 inhibited JNK catalytic activity, this would also be expected to decrease serine 63 and 73 phosphorylation. NIC-1 also did not affect Fos protein levels (Fig. 9A), inconsistent with a mechanism in which NIC-1 decreases AP-1 activity by reducing Fos expression.

One caveat of the Western blot experiments of Fig. 9A is that NIC-1 could potentially modulate temporal aspects of phosphorylation, and this might not be evident from steady-state measurements. We therefore examined the time course for phosphorylation of c-Jun (serine 73) and JunD upon TPA treatment of K562-Babe and K562-NIC-1 cells (Fig. 9B). NIC-1 had no effect on the time-dependent induction of phosphorylation, inconsistent with a role for NIC-1 in repressing AP-1 via disruption of signaling events necessary for activation of c-Jun and Jun family members. The failure of NIC-1 to inhibit c-Jun and JunD phosphorylation is consistent with the results of Fig. 8 showing that AP-1 repression requires nuclear localization of NIC-1. Because MAPK activation occurs in the cytoplasm, presumably NIC-1/NES, which localizes in part to the cytoplasm, would be competent to repress AP-1 if disrupted MAPK activation was involved.

**NIC-1 Does Not Inhibit AP-1 DNA Binding in Vitro**—In addition to the phosphorylation of serines 63 and 73 of c-Jun, which is required for transactivation, phosphorylation of c-Jun near the DNA binding domain has been reported to inhibit DNA binding (43). Dephosphorylation would be required to confer high-affinity DNA binding. It was important to test whether this mode of regulation is relevant to the NIC-1-mediated repression of AP-1 because NIC-1 could potentially antagonize dephosphorylation or potentiate phosphorylation, thereby inhibiting DNA binding and transactivation. K562-Babe and K562-NIC-1 cells were treated with TPA to activate AP-1, and nuclear extracts were isolated to measure AP-1 DNA binding activity by electrophoretic mobility shift assay. AP-1 DNA binding activity was strongly induced upon treatment of the cells with TPA, and there were no apparent qualitative or quantitative differences in the AP-1 complexes formed with extracts from K562-Babe and K562-NIC-1 cells (Fig. 10). Both anti-c-Jun and anti-c-Fos antibodies reduced the levels of complex formed, strongly arguing that the complex contains c-Jun and c-Fos subunits. To ensure that AP-1 components were not dephosphorylated upon nuclear extract isolation, phosphatase inhibitors were included in buffers, and this did not influence the AP-1 complexes, nor did it reveal an influence of NIC-1 on DNA binding. Thus, AP-1 complexes from K562-NIC-1 cells have an apparently normal DNA binding activity in vitro, suggesting that impaired AP-1-dependent transactivation is not caused by defective DNA binding. Furthermore, this result is inconsistent with an effect of NIC-1 on the levels of Jun or Fos family members because reduced levels of these AP-1 components should be evident by reduced AP-1-DNA complex formation.

**Physiological and Mechanistic Considerations of Notch-AP-1 Cross-talk**—AP-1 is essential for transcriptional activation of genes encoding numerous cytokines and enzymes mediating extracellular matrix remodeling, thereby establishing a critical role for AP-1 in immune and inflammatory responses (33, 34). A role for Notch signaling in immunity and vascular remodeling has emerged from recent genetic, molecular, and biochemical analysis (27, 73–75). Because the negative cross-talk between NIC-1 and AP-1 was evident in multiple cell types (Figs. 1 and 4), endogenous AP-1 target genes were affected (Fig. 5), and similar concentrations of NIC-1 were required for CSL-dependent transcription and AP-1 repression (Fig. 6), it seems reasonable to assume that such cross-talk would occur in diverse physiological contexts. Thus, it is attractive to hypothesize that negative cross-talk between Notch and AP-1 pathways would have important implications for immunity, inflammation, vascular remodeling, and potentially other physiological processes.

Establishing the physiological implications of the Notch-AP-1 cross-talk may be facilitated by further analysis of the underlying mechanisms. Two models to explain the NIC-1-mediated repression include disruption of AP-1 complex assembly on the chromatin template and impaired coactivator utilization by the AP-1-containing nucleoprotein complex. Given the overlapping sequence determinants for activation and repression, it is possible that the RAM domain interacts
with CSL to confer both activities; the only function previously ascribed to sequences within the RAM domain is CSL binding. Alternatively, because amino acids 1842–1848 of the RAM domain are selectively required for activation but not repression, one cannot rule out the possibility that the RAM domain interacts with a unique target to confer repression. The possibility of a distinct target mediating AP-1 repression is reinforced by the observation that NIC-1(−2105–2114) associates with CBP1 but does not repress AP-1. Thus, CBF1 binding is not sufficient to confer AP-1 repression. We asked whether CSL is required for AP-1 repression using CBF1-null OT11 cells (69) of AP-1 luciferase reporter plasmids. However, these cells were defective in TPA-induced AP-1 activation of AP-1 luciferase reporter plasmids and therefore were not useful for addressing this question.

AP-1 is known to be repressed by steroid hormone signaling pathways (62, 76–80). The mechanism of AP-1-steroid receptor cross-talk has required extensive analysis but remains incompletely understood. Nevertheless, it is instructive to compare the influence of steroid receptors and NIC-1 on AP-1. Recently, it was shown that repression of AP-1-mediated transactivation of the collagenase 3 promoter by the ligand-activated glucocorticoid receptor occurs after AP-1 DNA binding (79). The glucocorticoid receptor-interacting coactivator GRIP1 was important for AP-1 repression and it was proposed that GRIP1 confers activation and repression of target genes in a context-dependent manner. Based on the failure of NIC-1 to inhibit JNK-dependent phosphorylation of serines 63 and 73 of c-Jun (Fig. 9) and its lack of effect on AP-1 DNA binding in vitro (Fig. 10), the mechanism of AP-1 repression may be analogous to the glucocorticoid receptor scenario, whereby coactivator usage after DNA binding is an important determinant. AP-1 is known to utilize multiple coactivators including CBP/p300 (76) and Jab1 (47, 50). Preliminary experiments show that CBP overexpression does not overcome NIC-1-mediated repression of AP-1, suggesting that NIC-1 does not simply sequester limiting amounts of CBP.

Jab1 is a component of the COP9 signalosome (81), which has been implicated in multiple regulatory functions including the control of protein degradation. An influence of NIC-1 on COP9 signalosome-dependent AP-1 activation and more generally on COP9 signalosome function would have broad implications far beyond the control of AP-1 target genes.

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