RESEARCH COMMUNICATION

Notch activation stimulates transient and selective binding of Su(H)/CSL to target enhancers

Alena Krejčí and Sarah Bray

Department of Physiology Development and Neuroscience, University of Cambridge, Cambridge CB2 3DY, United Kingdom

The CSL [CBF1/Su(H)/Lag2] proteins [Su(H) in Drosophila] are implicated in repression and activation of Notch target loci. Prevailing models implicate the binding of CSL to DNA as the key regulatory step underlying the Notch signaling pathway. However, a more dynamic model has recently been proposed that suggests that CSL can bind to DNA as part of a larger complex, whose functional Su(H) sites have been shown to be transiently released during Notch activation. This implies that CSL can transiently bind to DNA to form a stable complex, which is then rapidly released and forms a ternary complex with DNA-bound factors. This model suggests that CSL can be more easily dissociated from DNA than previously thought.

Results and Discussion

EDTA triggers Notch activation in S2-N cells

To investigate changes in chromatin that accompany Notch activation, we needed to establish conditions where receptor activation could be temporally controlled. It has been reported that exposing cells to EDTA stimulates shedding of the Notch ectodomain [Rand et al. 2000; Gupta-Rossi et al. 2001]. This renders the residual transmembrane fragment a substrate for γ-secretase cleavage and, hence, results in Notch activation. Despite results suggesting that cell surface Notch in Drosophila would not be susceptible [Kidd and Lieber 2002], we have found that EDTA causes robust activation of E(spl) Notch-target genes within the E(spl) complex.

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The Notch signaling pathway plays an important role in a wide diversity of developmental contexts [Artavanis-Tsakonas et al. 1999; Schweisguth 2004; Bray 2006]. As the pathway impinges directly on the nucleus, it is important to determine the mechanisms that underlie the transcriptional response and how this response is tailored appropriately to different developmental contexts. Activation of Notch precipitates a proteolytic cleavage, catalyzed by the γ-secretase/presenilin complex, which releases an intracellular fragment called the Notch intracellular domain (NICD). This domain is stabilized in the cytoplasm by the adapter protein Mastermind (Mam) and serves as a tether for additional repressors, such as Groucho and CtBP, along with histone deacetylases (HDACs) [Kao et al. 1998; Morel et al. 2001; Barolo et al. 2002; Nagel et al. 2005; Oswald et al. 2005].

According to current models, CSL remains bound to the DNA at its targets, and the switch between repression and activation is mediated through exchange of associated proteins [Barolo et al. 2002; Bray 2006]. However, other transcription factors that participate in both repression and activation, such as the glucocorticoid receptor, appear to have a much more dynamic interaction with DNA [Agresti et al. 2005; Bosisio et al. 2006]. This equilibrium is altered by the presence of ligands, and it has been proposed that transcriptionally productive complexes have slower dissociation kinetics [Bosisio et al. 2006]. Such a model suggests that there is a rapid exchange of DNA-bound factors on and off the DNA, with stabilization occurring only as a consequence of recruiting secondary factors. If this were to apply in the case of CSL, it opens up the possibility that there could be distinct complexes (activation and repression) formed off the DNA, alleviating the need for NICD to actively dissociate a stable interaction between corepressors and CSL.

Here, we set out to monitor CSL/Su(H) occupancy at target enhancers under different conditions to ascertain whether it changes after Notch activation as predicted by the more dynamic models. We also investigated whether epigenetic modifications at target-genes correlate with inducibility and/or activation. To address these questions, we used a simple procedure to activate Notch in Drosophila cells, allowing stringent analysis of chromatin before and after activation, and assayed the 11 well-characterized Notch target genes within the E(spl) complex, whose functional Su(H) sites have been mapped [Fig. 1A; Bailey and Posakony 1995; Lecourtois and Schweisguth 1995; Nellesen et al. 1999; Cooper et al. 2000; Lai et al. 2000; Castro et al. 2005]. Our results demonstrate that Su(H) is only present at the subset of E(spl) enhancers that are transcriptionally active in a given cell type. More importantly, we detect a dramatic and transient increase in Su(H) occupancy after Notch activation, in agreement with dynamic models of gene regulation.

Keywords: Notch, chromatin, developmental signaling, transcription factor, CSL proteins, E(spl) complex

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Figure 1. EDTA elicits Notch activation in S2-N cells. [A] Diagram of \(E\text{(spl)}\) complex. Genes are indicated by arrows: basic helix-loop-helix genes [blue], Bearded-type [gray], not Notch responsive [white]. [B] mRNA levels of \(E\text{(spl)}\) genes before and 30 min after EDTA treatment of S2-N cells. Bottom panel shows results plotted on a more sensitive scale. (C) \(m3\) and \(m7\) mRNA levels in EDTA-treated S2-N cells with \(\gamma\)-secretase inhibitor DFK-167 [300 µM]. [D, top panel] Su[H] is detected as two bands in the total input and in \(\alpha\)-Su[H] immunoprecipitates with and without EDTA treatment. [Bottom panel] Nicd is only present in the immunoprecipitated sample from EDTA-treated cells. Positions of molecular weight markers (in kilodaltons) are indicated for the bottom panel.

Figure 2. \(\text{Su(H)}\) occupancy increases after Notch activation. ChIP with \(\alpha\)-Su[H] [A,C] or \(\alpha\)-Pol II [phosphorylated CTD] [B,D] antibodies in S2-N [A,B] and DmD8 [C,D] cells before [orange] or 30 min after [blue] EDTA. Precipitated DNA was quantified by real-time PCR. Each gene is represented by two or three fragments. [s] Su[H]-binding/enhancer region; [p] promoter; [o] ORF. A single \(5^\prime\) region was amplified when Su[H] sites were close to the promoter. Control was rpd4 ORF [p]. Results are average of three independent experiments (error bars indicate standard error of the mean). Increase in enrichment after activation of \(m3\)-s \(P = 0.03\) and \(m3\)-s \(P = 0.003\) in S2-N cells and of \(m6\)-s \(P = 0.04\), \(m7\)-s \(P = 0.04\), \(m3\)-s \(P = 0.003\), \(m6\)-s \(P = 0.002\), and \(m7\)-s \(P = 0.02\) in DmD8 cells is significant.
Su[H] repressor complex for their repressed state. In agreement, only m3 and mβ were markedly derepressed when Su[H] or the corepressor Hairless were depleted using RNA interference [Supplementary Fig. S1D].

The distributions of Su[H] and Pol II were much more widespread across the E(spl) complex in DmD8 cells. In general, enhancers that were enriched in the Su[H] ChIP corresponded to genes where Pol II was recruited [Fig. 2C,D]. Two genes, m4 and m5, were not enriched in the Su[H] ChIP nor the Pol II ChIP, and neither did we detect any mRNA increase after Notch stimulation [Fig. 2D; data not shown]. We note that m3a was significantly enriched in the Pol II, but not the Su[H] ChIP. This discrepancy may indicate that our PCR-amplified fragment does not encompass the functional Su[H] site for m3a. Otherwise, as in S2-N cells, there was a good correlation in DmD8 cells between Su[H] recruitment and the presence of Pol II after activation. What was surprising, however, was that there was little enrichment for Su[H] at the inducible genes prior to Notch activation. Most enhancers were only detectably enriched in the Su[H] ChIP after activation.

**Su[H] occupancy is enhanced after Notch activation**

Current models imply that Su[H] levels at target loci will be unchanged on Notch activation, as they postulate an exchange between Nicd and corepressors. The significant increase in Su[H] occupancy following Notch activation was therefore unexpected [Fig. 2A, C. cf. blue and orange bars]. In S2-N cells, there was a 2–2.5 times increase in enrichment for m3 and mβ enhancer fragments after activation. Similar results were obtained using α-GFP to perform ChIP in S2-N cells expressing a GFP-Su[H] fusion [data not shown]. In contrast, no increase in occupancy was detected following EDTA treatment of S2 cells without Notch [Supplementary Fig. S3] nor in ChIPs against other chromatin-associated factors [e.g., H3, H2A.v] [Fig. 4, below; Supplementary Fig. S4] in S2-N cells, confirming that the increased ChIP of the enhancers with Su[H] is a specific effect. In DmD8 cells, the increase in Su[H] occupancy after activation was even more dramatic [Fig. 2C]. Enrichment at five of the enhancers significantly increased in the ChIP after activation, with a >10-fold increase in occupancy at m6, mα, and mαβ [Fig. 2C].

The increased enrichment of target loci following Notch activation suggests that the activation complex is more stably associated with chromatin. A time course of m3 mRNA levels in S2-N cells post-induction showed that expression declines once activation ceases [Fig. 3A]. To ask whether the increase in Su[H] occupancy following Notch activation was also transient, we compared chromatin from activated S2-N cells (30 min) and from cells after activation had ceased [180 min] [Fig. 3B]. There was a robust increase in enrichment of the m3 enhancer in ChIP from activated cells at 30 min, but by 180 min, levels returned to those in uninduced cells. These data indicate that the association of Su[H] with its target enhancers in S2-N cells is more dynamic than has been proposed and is stimulated by Notch activation.

To confirm that the increased Su[H] occupancy is also transient in DmD8 cells and to determine whether it can be attributed to the formation of complexes with Nicd, we performed α-Su[H] and α-Nicd ChIP at different times after activation and assayed the levels of the m7 enhancer fragment [Fig. 3C,D]. In both ChIPs there was a transient increase in enrichment of the m7 enhancer at 30 min, with the levels declining over subsequent time points. Furthermore, analysis of the amount of Nicd that coimmunoprecipitated with Su[H] showed peak levels at 30–60 min post-activation [Fig. 3E]. Interestingly, Nicd appeared as a smear of increasing size, suggesting that it acquires post-translational modifications with time. Together, these data support the hypothesis that the increased Su[H] occupancy post-activation occurs as a consequence of its association with Nicd.

In previous studies, high levels of the Nicd fragment were found to promote nuclear accumulation of Su[H] [Fortini and Artavanis-Tsakonas 1994; Kidd et al. 1998]. We therefore investigated whether the more physiological levels of Nicd produced by EDTA treatment could influence Su[H] distribution in S2-N cells. Staining of cells before and after EDTA treatment revealed a significant shift toward nuclear localization of Su[H] in EDTA-treated S2-N cells [Fig. 2F,H] compared with mock treated cells [Fig. 2F,G]. No such shift was seen in control cells [Fig. 2F, Supplementary Fig. S3]. Activation of Notch was accompanied therefore by an increase in the nuclear concentration of Su[H] as well as by increased occupancy of target enhancers. This brings into question whether some interactions between Nicd, Su[H]/CSL,
and Mam could occur before nuclear entry, explaining why the activation complex has been detected in cytoplasmic fractions (Jeffries et al. 2002).

Chromatin marks associated with highly inducible genes

Reversible post-translational modifications on histone tails and exchange of histone variants play an important role in the regulation of transcription. We therefore investigated whether the difference in Su(H) binding across the 11 E(spl) genes could be related to specific chromatin modifications. ChIP was performed on chromatin from S2-N and DmD8 cells before and 30 min after Notch activation using antibodies directed against histone H3, against histone modifications primarily associated with active chromatin (trimethylation at Lys 4 of histone H3 [\textit{\textsuperscript{tri}-meK4-H3} and acetylation of histone H4 \textit{[AcH4]}], and against the histone variant \textit{H2A.v}. In S2-N cells there was a clear correlation between the chromatin modifications and high Su(H) occupancy (Fig. 4A,B, Supplementary Fig. S4A,B). In DmD8 cells this correlation was less robust, although many of the same trends were evident (Fig. 4C,D, Supplementary Fig. S4C,D).

One characteristic observed in both cell types was a reduced association of H3 and by implication of nucleosomes with the active genes. In S2-N cells this was evident prior to Notch activation, with a lower than average enrichment of both m3 and m\textsuperscript{\beta} enhancer/promoter in the H3 ChIP (Fig. 4A). In DmD8 cells these two genes also had lower H3 coverage prior to activation (Fig. 4C), and there was a further decrease in H3 at these and at five other genes (\textit{m7}, \textit{ma}, \textit{m2}, \textit{m6}, \textit{m7}) after activation. Thus, activation is accompanied by a decrease in H3, and by implication, with a loss of nucleosomes from the enhancer/promoter regions. Both cell types also showed a large increase in the proportion of \textit{AcH4} at the active genes, following Notch activation (Fig. 4B,D). This fits with reported recruitment of a HAT [p300] to the Nicd/CSL/Mam complex (Oswald et al. 2001; Wallberg et al. 2002). However, in both cell-types, \textit{AcH4} encompassed adjacent loci that were not highly expressed, suggesting either that this complex can contact histones at a distance from its initial site of recruitment or that there is secondary recruitment of other HATs.

Two other chromatin marks that correlated strongly with activated genes in S2-N cells were \textit{tri}-meK4-H3 (Supplementary Fig. S4A) and enrichment for the histone variant H2A.v/H2A.Z (Supplementary Fig. S4B), which is suggested to mark transcriptionally poised genes (Raisner et al. 2005; Zhang et al. 2005). In DmD8 cells the correlation with \textit{tri}-meK4 was less evident, but four out of eight induced genes showed significant enrichment (Supplementary Fig. S4C). Likewise, there was no specific enrichment for H2A.v prior to activation (Supplementary Fig. S4D). However, in both S2-N and DmD8 cells, the proportion of H2A.v decreased at activated loci following EDTA (Supplementary Fig. S4E). A similar decrease in H2A.Z was found to accompany activation of the mammalian \textit{c-myc} gene, where removal of H2A.Z was proposed to facilitate activation (Farris et al. 2005). The observed decrease in H2A.v following Notch activation is in agreement with this model, although more widespread analysis will be needed to determine whether it is indicative of a general mechanism at Notch targets.

Concluding remarks and mechanistic implications

Our results demonstrate that there is a significant increase in Su(H) occupancy at target genes following EDTA/Notch activation. This increase is transient and correlates with the presence of Nicd, implying that the kinetics of binding differ when Su(H) is complexed with Nicd, and that the association between Su(H) and its cognate sites is much more dynamic than expected. We also note that there are differences in Su(H) occupancy between genes prior to activation, suggesting the possibility of gene-specific modes of regulation [i.e., at some there may be constitutive recruitment of Su(H), whereas at others Su(H) binding is only signaling induced].

Several mechanisms could account for the increased Su(H) occupancy after Notch activation. One possibility is that the activation complex has a higher affinity for DNA. Structural analysis of the CSL/Nicd/Mam ternary complex did not reveal any novel interactions between

Figure 4. Chromatin modifications across \textit{E(spl)} genes before and after Notch activation in S2-N and DmD8 cells. ChIP was performed with the indicated antibodies in S2-N [\textit{A,B}] and DmD8 [\textit{C,D}] cells before (orange) and 25 min after (blue) EDTA and bound fragments were quantified by real-time PCR. Results are an average of three independent experiments (error bars indicate standard error of the mean). (\textit{A,B}) For each column, levels were first calculated relative to input and then as a ratio to the equivalent sample from the \textit{\alpha}H3 ChIP Control were \textit{rp49} (rp). In \textit{\alpha}H3 ChIP, the decrease in enrichment of \textit{mb6} (\textit{P} = 0.02) and \textit{m3} (\textit{P} = 0.05) in S2-N cells and of \textit{m6} (\textit{P} = 0.006), \textit{m6} (\textit{P} = 0.03), \textit{m6} (\textit{P} = 0.004), and \textit{m7} (\textit{P} = 0.03) in DmD8 cells are all significant.
S2-N cells are a stable Notch-expressing S2 cell line containing a Cu²⁺-inducible [metallothionein promoter] pMT-Notch construct (Fehon et al. 1990) and grown under permanent selection with 10 μM methotrexate (Sigma). Expression of full-length Notch was induced overnight by 600 µM CuSO₄ (Sigma) in cell culture medium. DmD8 cells were obtained from the Drosophila Genomics Resource Center (http://dgrc.cgb.indiana.edu). Activation in S2-N and DmD8 cells was achieved by exposing cells to 2 mM EDTA in PBS for 30 min. This was followed in some cases by replacement of cell culture medium and a further period of incubation. Control cells were treated with PBS without the addition of EDTA (mock-treated).

RNA isolation and quantification
RNA was isolated by Trizol (Ambion). Reverse transcription was performed with M-MLV RT (Promega) and oligo-dT primers or random hexamers [Promega]. cDNA levels were quantified by real-time PCR using QuantiTec Sybr Green PCR mix (Qiagen) and the ABI Prism machine. The calibration curve was constructed from serial dilutions of genomic DNA, and values for all genes were normalized to the levels of rp49. To allow comparison among primer sets, a constant amount of genomic DNA [standard DNA] was used in each PCR run for additional normalization.

ChIP
Cells were cross-linked with 2% formaldehyde, the reaction was quenched by 0.125 M glycine, and the cells were harvested, washed with PBS, and resuspended in nuclear lysis buffer [the composition of ChIP buffers is provided in the Supplemental Material]. Lysate was diluted 10 times in immunoprecipitation dilution buffer, sonicated by Bioruptor (Diagenode), and precleared with rabbit IgG (Sigma) and protein G agarose (Santa Cruz Biotechnology). ChIP reactions were performed overnight at 4°C with 700 µL of lysate and 1–2 µg of specific antibodies. Immunocomplexes were isolated with Protein G agarose, washed twice with wash buffer1 and twice with wash buffer2, and then de-cross-linked at 65°C in 0.25 M NaCl. Samples were treated with 0.2 mg/mL proteinase K, extracted with phenol/chloroform, and ethanol-precipitated. Pellets were resuspended in 35 µL of water and 0.5 µL was used for real-time PCR [primer sequences available on request]. To allow comparison among primer sets, a constant amount of total input genomic DNA was used in each PCR run for normalization. The following antibodies were used: acetylated H4 [Upstate Biotechnology, 06-866], histone H3 (Abcam, ab1791), trimethyl K4 [Abcam, ab8580], CTD-phosphorylated Pol II (Abcam, ab5408), Su(H) [Santa Cruz Biotechnology, sc5813], Nicd [Developmental Studies Hybridoma Bank, C17.9G0], and H2A.v [gift from R.L. Glaser, New York State Department of Health, Albany, NY].

Immunostaining
Cells were grown on glass coverslips, with or without Cu induction, and mock-treated or activated by EDTA, then fixed for 30 min with 4% PFA. After blocking with PBT (1× PBS, 0.2% Triton X-100, 5% BSA) for 30 min, cells were stained with primary antibody for 90 min, washed with PBT, and stained with secondary antibody for 90 min.

Western blotting
Immunoprecipitated proteins were resolved by 7% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with specific antibodies as indicated. Bound antibodies were detected using horseradish-conjugated secondary antibodies and the ECL detection system (Amersham).

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