Recognition and Ubiquitination of Notch by Itch, a Hect-Type E3 Ubiquitin Ligase

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Running title: Ubiquitination of Notch by Itch E3 ligase
ABSTRACT

Genetic studies identified Itch, a Hect domain-containing E3 ubiquitin ligase, that is disrupted in non-agouti lethal mice or Itchy mice. Deficiency of Itch displays abnormal immune responses and constant itching in the skin. Here, Itch was shown to associate with Notch, of the latter being involved in cell fate decision in many tissues of mammals including the immune system. Itch binds to the N-terminal portion of Notch intracellular domain via its WW domains and promotes ubiquitination of Notch through Hect protein ligase domain. Thus, Itch may participate in the regulation of immune responses by modifying Notch-mediated signaling.
INTRODUCTION

Ubiquitin (Ub)-dependent protein conjugation has been implicated in a variety of cellular processes, including cell-cycle control, signal transduction, transcriptional regulation, DNA repair, receptor down-regulation, antigen presentation, and apoptosis (1-3). Abnormalities in the Ub system have been shown to cause pathological responses, including malignant transformation, and several genetic diseases (4). Ubiquitination of protein substrate involves a cascade of enzymatic reactions: first, Ub, a highly conserved 76-amino acid polypeptide, is activated by Ub-activating enzyme, or E1, leading to an ATP-dependent formation of high energy thiol-ester bonds between the C-terminus of Ub and E1; the activated Ub is then transferred to E2s (Ub-conjugating enzymes or Ubcs). E2 enzymes then mediate the transfer of Ub to the target protein directly, or to E3 Ub protein ligases, which are responsible for substrate recognition and for promoting Ub ligation to substrate. A substrate may be multiply ubiquitinated by sequential linkage of additional Ub molecules to form a poly-Ub chain, which marks the protein substrate for the recognition and consequent degradation by 26S proteasome. The E3s are the critical components that provide specificity to the Ub system by direct interaction with substrate. Itch, a novel E3 Ub ligase, is absent in the non-agouti-lethal 18H mice or Itchy mice (5). These mice develop immunological and inflammatory diseases, including inflammation in the lung and stomach, hyperplasia in lymphoid cells and hematopoietic cells, and constant itching in the skin (6), suggesting that Itch is involved in the regulation of immune responses. Itch contains an N-terminal protein kinase C-related C2 domain, four WW domains and C-terminal Hect (Homologous to the E6-associated protein carboxy terminus) ligase domain (5). At present, the biological pathways affected by Itch mutation have not been identified.

A recent genetic study showed that suppressor of deltex [Su(dx)], an Itch-related protein in Drosophila melanogaster, negatively regulates Notch-mediated signaling (7), suggesting that Notch may be a target protein for Itch. Notch proteins are a family of evolutionarily conserved transmembrane receptors that have been shown to be important regulators in cell fate decisions in many tissues of mammals including the immune system (8). Notch signaling is critically involved in the induction of T cell lineage development.
and T cell survival (9,10). We wished to understand whether Itch may induce Notch-Ub conjugation and thus regulate Notch-mediated signaling pathways in T cells.

MATERIALS AND METHODS

**Plasmids**--Protein expression in Jurkat T cells uses a mammalian vector pEFneo with none, Myc or HA epitope tag (11). Notch-ΔEF cDNA encoding the transmembrane and entire intracellular domain of murine Notch-1, extending from I1701 to K2530. The intracellular domain of Notch-1 contains an N-terminal transcriptional factor binding domain, six ankyrin repeat, and a C-terminal PEST sequences (12). Notch-ΔPEST cDNA derived from Notch-ΔEF with the C-terminal PEST sequences deleted, encoding from I1701 to E2480, were provided by B. Yankner. Notch-ΔEN encoding N-terminal transcriptional factor binding domain and the six ankyrin repeats, from V1744 to D2097, was amplified by PCR and the construct was confirmed by sequencing. HA-Ub cDNA encodes eight ubiquitin repeats individually tagged with an HA epitope (13), provided by M. Treier, was subcloned into pEFneo. Mouse Itch cDNA was from N. G. Copeland. Mutation at the putative active-site cysteine 822 to alanine was generated by site-directed mutagenesis. For the protein expression in bacteria, Itch cDNAs lacking the N-terminal C2 domain (ΔC2; extending from N226 to E854), the four WW domains (WW; from P268 to P471), or the C-terminal Hect domain (Hect; from Q472 to E854), was subcloned into a pGEX GST fusion vector.

**Cell culture and transfection**-- Simian virus 40 T antigen-transfected human leukemic Jurkat T cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum and antibiotics. For protein expression in Jurkat T cells, cells were transfected with appropriate amounts of plasmids (usually 3–10 µg total) by electroporation (240V, 960 µF; Bio-Rad). Cells were lysed in Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM NaPiP, 5 mM NaF, 2 mM NaVO₄, 10 µg/ml each aprotinin and leupeptin) for 10 min at 4°C and insoluble materials were removed by centrifugation at 15,000 x g (4°C for 10 min).
**Immunoprecipitation and pull-down assay**-- For immunoprecipitation, lysates (~1 x 10^7 cells) were mixed with antibodies (1 µg) for 2 hr, followed by addition of 40 µl protein A/G Plus-Sepharose beads (Santa Cruz Biotechnology) for an additional hr at 4°C. Immunoprecipitates were washed four times with NP-40 lysis buffer, and boiled in 20 µl 2X Laemmlí's buffer. Samples were subjected to SDS/8% polyacrylamide gel electrophoresis (PAGE) analysis and electrotransferred onto nitrocellulose membranes. Membranes were probed with the indicated primary antibodies (1 µg/ml), followed by horseradish peroxidase-conjugated secondary antibodies. Membranes were then washed and visualized with enhanced chemiluminescence (ECL) detection system (Amesham). When necessary, membranes were stripped by incubation in 62.5 mM Tris-HCl, pH 6.7/100 mM 2-mercaptoethanol/2% SDS for 1 hr at 70°C with constant agitation, washed, and then reprobed with other antibodies as indicated.

GST fusion proteins were expressed in E. coli BL21 (DE3) by induction with 0.5 mM isopropyl-b-D-thiogalactopyranoside for 2 hr at room temperature and purified as described (14). For the pull-down assay, GST fusion proteins (5 µg) were added to the cell lysates for incubation at 4°C for 2 hr, followed by addition of 50 µl of glutathione-Sepharose beads (Pharmacia) for 30 min. Precipitates were washed four times with the lysis buffer and subjected to SDS-PAGE (10%) and immunoblotting with indicated antibodies.

**In vitro ubiquitination assay**-- His-tagged Ub, E1, Ubc6 or GST-tagged UbcH7 were prepared using standard protocol as described (14). Ubiquitination reactions (15 µl) contained His-E1 (100 nM), His-Ubc6 or GST-Ubc7 (0.5 µM), GST-ΔC2 or GST-ΔC2-CA (5 µg), ubiquitin (5 µM), and ATP (2 mM) in Ub buffer [50 mM Tris-HCl (pH 7.5), 2.5 mM MgCl₂, and 0.5 mM DTT] with immunoprecipitated Notch-ΔEF on the Sepharose beads as substrate. Incubation was at 25°C for 90 min. Reactions were stopped by washing the beads four times with lysis buffer. The beads were resuspended in SDS sample buffer, containing 2% SDS and 50 mM DTT. The reaction mixtures were resolved by SDS-PAGE (8%), transferred onto nitrocellulose membrane and subjected to immunoblotting with anti-Myc antibody. The same membrane was reprobed with anti-Ub antibody.
Generation of anti-Itch polyclonal antibody-- Two New Zealand White rabbits were immunized with GST-ΔC2 fusion protein. Antisera were absorbed with excess amounts of GST protein bound on glutathione-Sepharose beads to remove antibodies against GST. The antisera recognized an ~110 kDa protein in Jurkat T cells and mouse thymocytes by immunoprecipitation and immunoblotting at a dilution of 1:500.

RESULTS AND DISCUSSION

To determine whether Notch is ubiquitinated, we expressed a Myc epitope-tagged Notch construct containing its transmembrane and intracellular domains (Notch-ΔEF) in the absence or the presence of HA epitope-tagged Ub in human Jurkat T cells. Co-expression of HA-Ub with Notch-ΔEF caused the formation of higher molecular weight smear in the anti-Myc immunoprecipitates, that were recognized by anti-HA antibody (Fig. 1a). Addition of MG132, a protease inhibitor, which prevent 20S proteosome-mediated proteolysis (15), enhanced this event. The Notch-Ub conjugate formation was further enhanced by the addition of UbcH7, an E2 enzyme (Fig. 1b).

We tested whether Itch is involved in ubiquitination of Notch. Co-expression of Itch with Notch-ΔEF in Jurkat T cells markedly induced Notch-Ub conjugation, which was further increased by MG132 treatment (Fig. 1c). The Notch-Ub reaction was then reconstituted in vitro. To this end, we generated glutathione-S-transferase (GST) fusion protein containing the N-terminal C2 domain-truncated Itch (ΔC2). Immunopurified Notch-ΔEF served as substrate. Ubiquitination reactions were initiated by addition of Ub, human E1, UbcH7 E2, and ATP in the presence of GST or GST-ΔC2. The ubiquitinated products were analyzed by immunoblotting with anti-Myc antibody, and then reprobed anti-Ub antibody (Fig. 1d). Incubation of Notch-ΔEF with GST-ΔC2, but not with GST, led to the formation of slower migrating proteins what were recognized by anti-Myc antibody (left panel). This higher molecular forms of Notch-ΔEF represented differential poly-ubiquitination, as revealed by reprobing the same membrane with anti-Ub
antibody. We conclude that Notch intracellular domain is ubiquitinated and that it serves as a direct substrate for Itch E3 ligase.

It is generally defined that E3 ligases interact directly with protein substrates and facilitate the transfer of E2-loaded Ub to the target proteins (16). The interaction between Itch and Notch was examined by pull-down assay using GST-ΔC2, GST fusion protein containing the four WW domains (GST-WW), or the C-terminal Hect domain (GST-Hect), or GST alone. Lysates prepared from untreated or pervanadate-treated Jurkat T cells overexpressing Notch-ΔEF were incubated with respective GST fusion proteins and the precipitates were analyzed by anti-Myc antibody (Fig. 2a). GST-ΔC2, which encompasses the four WW domains and the Hect domain of Itch, bound to Notch. GST-WW, but not GST-Hect nor GST alone precipitated Notch to a similar degree as GST-ΔC2 (top panel). We have previously shown that treatment of Jurkat T cells with pervanadate induces phosphorylation at both the tyrosine and serine/threonine residues such as in Cbl adaptor protein (17), we then tested whether similar treatment could affect the phosphorylation state of Notch and thus the association between Notch and Itch. However, treatment of Jurkat T cells with pervanadate did not affect the interaction.

Notch has C-terminal proline (P)-glutamate (E)-serine (S)-threonine (T)-rich (PEST) sequences, which are implicated in protein degradation (18). We tested whether removal of the PEST region (Notch-ΔPEST) affects the association between Itch and Notch. Lysates prepared from Jurkat T cells expressing Notch-ΔPEST or Notch-ΔEF were incubated with GST, GST-WW or GAT-ΔC2 and the precipitates were analyzed by immunoblotting with anti-Myc antibody. Deletion of the PEST sequences in Notch did not affect the interaction between Itch and Notch (bottom panel). The in vitro association was confirmed by in vivo co-immunoprecipitation assay. Itch was co-expressed with Notch-ΔPEST or Notch-ΔEF in Jurkat T cells and the cell lysates were immunoprecipitated with anti-Itch antibody. Both Notch-ΔPEST and Notch-ΔEF were co-immunoprecipitated with Itch (Fig. 2b).

The Ub conjugation to Notch-ΔEF or to Notch-ΔPEST was compared in Jurkat T cells. The two proteins were ubiquitinated to a similar degree under resting conditions, that was enhanced by MG132.
treatment (Fig. 2c). Like Notch-ΔEF, co-expression with Itch remarkably increased ubiquitination of Notch-ΔPEST (Fig. 2d).

We tried to further map the Itch binding region in Notch intracellular domain. A Myc-tagged cDNA construct was generated that contains N-terminal RAM transcription factor binding domain and the six ankyrin repeats (Notch-ΔEN). Notch-ΔEN as well Notch-ΔEF was expressed in Jurkat T cells. Cell lysates were incubated with GST or GST-WW and the precipitates were analyzed by immunoblotting with anti-Myc. GST-WW but not GST alone still bound to Notch-ΔEN, although less efficiently than to Notch-ΔEF (Fig. 3a). Thus, the N-terminal region of Notch intracellular domain is responsible for the association with the WW domains of Itch.

The Ub conjugation in Notch-ΔEN was examined. Ubiquitination of Notch-ΔEN was detected in cells co-expressing with HA-Ub, that was greater upon MG132 treatment (Fig. 3b). The same cell lysates were immunoprecipitated with anti-HA antibody and the immunoprecipitates were analyzed with anti-Myc antibody. Mono- or poly-ubiquitinated Notch-ΔEN was present in the Ub immunoprecipitates (Fig. 3c).

To determine whether the N-terminal intracellular domain of Notch can serve as a substrate for Ub conjugation by Itch, Notch-ΔEN was co-expressed with Itch. Co-expression with Itch significantly increased ubiquitination of Notch-ΔEN (Fig. 3d), suggesting that this portion is required for both recognition and subsequent ubiquitination of Notch by Itch.

Hect domains of E3 ligases of this family share a region of ~350 amino acids in the C terminus, which contains E2 binding site and a putative active-site cysteine residue. The Hect domain governs the transfer of Ub from E2, to the putative active-site cysteine, and then to the protein substrate, and the poly-ubiquitination of the substrate (19). To evaluate a possible role of Itch Hect domain in mediating Notch ubiquitination, we next generated a construct in which the putative active-site cysteine 822 was mutated to an alanine (Itch-CA) and examined whether this mutation affects the ligase activity of Itch. Whereas wild-type Itch enhanced Ub conjugation to Notch-ΔEN under both resting and MG132-treated conditions, Itch-CA mutation abolished this effect (Fig. 4a). In fact, Itch-CA reduced ubiquitination of Notch-ΔEN to a
lower level as compared with cells transfected with empty control vector, suggesting that Itch-CA plays a dominant negative role.

The role of this putative active-site cysteine in promoting ubiquitination of Notch was also examined in vitro. Replacement of cysteine 822 with alanine in GST-ΔC2 of Itch fusion protein almost completely abolished the Ub conjugation to immunopurified Notch-ΔEF, as revealed by either anti-Myc or by anti-Ub blotting (Fig. 4b), suggesting that the putative active-site cysteine is indeed required for the transfer of Ub to the substrate.

In this study we demonstrated that Itch acts as an E3 protein ligase to induce Notch ubiquitination using both in vitro and in vivo ubiquitination assays. More importantly, Itch WW domains interact with Notch N-terminal region and a functional Hect ligase domain is required for promoting Ub conjugation to Notch. The results suggest that Notch may function as a physiological target for Itch E3 ligase.

WW domains derive their name from the presence of two highly conserved tryptophan (W) residues, which are spaced 20-22 amino acids apart. WW domains contain 38-40 amino acids in a triple-stranded β sheet and are found in proteins that participate in cell signaling or regulation. These domains are implicated in mediating protein-protein interactions by binding to proline-rich motifs (20) or phosphoserine- and phosphothreonine-containing elements in their binding partners (21). Notch has a proline-, serine-, and threonine-rich PEST sequence at its C-terminus. It is also known that PEST sequences in general is involved in protein stability (18). It is surprising to note that this sequence is not required for the interaction with Itch WW domains. Rather, it was shown that an N-terminal region is responsible for the interaction. This region contains ankyrin repeats and has been shown to mediate protein-protein interaction (8). Inspection of this region, however, does not show any proline- or serine/threonine-rich sequences. Further study is needed to understand whether this interaction is direct or through indirect factor(s).

Notch has been implicated in the regulation of T cell development and hematopoiesis, particularly in CD4 versus CD8 lineage decision (22-25). The results of this work suggest that Itch, an E3 protein ligase, recruits and promotes the Ub conjugation of Notch, thus down-regulating Notch-mediated signaling. This
notion is consistent with the observation in Drosophila: ectopic overexpression of Su(dx), an Itch homologue, enhances the phenotypes of loss-of-function mutations in Notch (7). Taken together, these data may support an idea that Itch or its homologues play an evolutionarily conserved role as a negative regulator of Notch receptor signaling. Further functional studies of Itch in T cell development and activation will provide molecular insights into the abnormal immune responses manifested in Itchy mice.
REFERENCES


Figure Legends:

**Fig. 1.** Ubiquitination of Notch intracellular domain. (a) Jurkat T cells (5 X10^7 cells/sample) were transfected by electroporation with plasmids containing a Myc-tagged Notch cDNA fragment encompassing the transmembrane domain and the entire intracellular domain (Notch-ΔEF) (1 µg each) without (lanes -) or with (lanes +) a plasmid containing HA-tagged ubiquitin (Ub). 48 hrs later, transfected cells were left untreated (lanes -) or treated (lanes +) with MG132 (50 µM) for 30 min. Cell lysates were incubated with anti-Myc antibody and the immunoprecipitates were resolved by SDS/PAGE (8%) and electrotransferred onto nitrocellulose membrane. The membrane was blotted with anti-HA antibody (top panel). The positions of poly-ubiquitinated Notch protein [(Ub)n-Notch-ΔEF] are indicated. The same membrane was reprobed with anti-Myc antibody. The position of Notch-ΔEF is indicated by arrow. (b) Cells similarly transfected as in (A) with plasmids containing Notch-ΔEF and HA-Ub, without (lanes -) or with (lanes +) the addition of UbcH7 E2 plasmid (1 µg each). Transfected cells were treated and analyzed as in (a). (c) Cells were transfected with plasmids containing Notch-ΔEF and HA-Ub, without (lanes -) or with (lanes +) Itch plasmid (1 µg each). Transfected cells were analyzed as in (a). (d) In vitro ubiquitination of Notch. Immunoprecipitated Notch-ΔEF on Sepharose beads was incubated with Ub, E1, E2, ATP in the presence of GST or GST-ΔC2 at 25°C for 90 min. The reaction mixture was extensively washed with lysis buffer and analyzed by immunoblotting with anti-Myc antibody (left panel). The same membrane was reprobed with anti-Ub antibody. The positions of poly-ubiquitinated Notch protein [(Ub)n-Notch-ΔEF] are indicated in both panels.

**Fig. 2.** Interaction of Itch with Notch. (a) Jurkat T cells were transfected with Notch-ΔEF plasmid (5 µg) and lysates prepared from untreated or pervanadate (PV)-treated cells were incubated with GST, or GST fusion proteins containing both four WW domains and Hect domain (ΔC2) or with WW domains or Hect domain only (5 µg each) for 2 hrs at 4°C. Precipitates were subjected to 10% SDS/PAGE and immunoblotting with anti-Myc antibody (top panel). The position of Notch-ΔEF is indicated. Bottom panel,
Jurkat T cells were transfected with plasmids containing a PEST sequence-deleted Notch (Notch-ΔPEST) or Notch-ΔEF. Cell lysates were incubated with GST, GST-WW or GST-ΔC2 and analyzed as in the top panel. (b) Jurkat T cells were co-transfected with Itch plasmid (5 µg) with pEF empty vector, Notch-ΔPEST, or Notch-ΔEF plasmid (5 µg each). Cell lysates were incubated with anti-Itch antibody and the immunoprecipitates were analyzed with anti-Myc antibody (top panel). The same membrane was reprobed with anti-Itch antibody. The position of Itch is indicated. (c) Notch-ΔEF or Notch-ΔPEST was expressed in Jurkat T cells together with HA-Ub. Ub conjugation to these proteins was analyzed as in Fig. 1A. (d) Notch-ΔPEST was co-expressed without (lanes -) or with (lanes +) Itch. Ubiquitination of Notch-ΔPEST was analyzed as in Fig. 1a.

Fig. 3. Itch binds to the N-terminal portion of Notch intracellular domain and promotes its Ub conjugation. (a) Jurkat T cells were transfected with a plasmid containing the N-terminal RAM and Ankyrin repeat domains (Notch-ΔEN) or with Notch-ΔEF plasmids (5 µg each). Cell lysates were incubated with GST or GST-WW protein (5 µg each). Precipitates were analyzed with anti-Myc antibody. The positions of Notch-ΔEN and Notch-ΔEF are indicated. (b) Cells were transfected with Notch-ΔEN plasmid (1 µg) without (lanes -) or with (lanes +) HA-Ub plasmid (1 µg) and the cells were analyzed as in Fig. 1A. (e) The same cell lysates were incubated with anti-HA antibody and the immunoprecipitates were analyzed with anti-Myc antibody. (d) Cells were transfected with Notch-ΔEN plasmid plus HA-Ub in the absence (lanes -) and the presence (lanes +) of Itch plasmid. Transfected cells were analyzed as in Fig. 1 a.

Fig. 4. Requirement of cysteine 822 for the Ub ligase activity of Itch. (a) Cells were transfected with Notch-ΔEN and HA-Ub plasmids plus either empty pEF vector, Itch, or an Itch cysteine to alanine mutant (Itch-CA) plasmid. Lysates prepared from cells untreated (lanes -) or treated with MG132 (lanes +) were immunoprecipitated with anti-Myc, blotted with anti-HA, and then reprobed with anti-Myc. (b) In vitro ubiquitination reactions were performed as in Fig. 1D, with GST as a control, GST-ΔC2 (ΔC2), or GST-
ΔC2-CA (ΔC2-CA) as an E3. The reaction mixtures were analyzed with anti-Myc antibody (left panel), and then reprobed with anti-Ub (right panel).
Fig. 1. Itch/Notch
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**Blot:** anti-Myc

**IP:** anti-Itch

**Reprobe:** anti-Itch

Fig.2 Itch/Notch
Fig. 2-cont.
Fig. 3. Itch/Notch
Fig. 4. Itch/Notch