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Lunatic fringe protein processing by proprotein convertases may contribute to the short protein half-life in the segmentation clock

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ABSTRACT

During vertebrate segmentation, oscillatory activation of Notch signaling is important in the clock that regulates the timing of somitogenesis. In mice, the cyclic activation of NOTCH1 requires the periodic expression of Lunatic fringe (*Lfng*). For LFNG to play a role in the segmentation clock, its cyclic transcription must be coupled with post-translational mechanisms that confer a short protein half-life. LFNG protein is cleaved and released into the extracellular space, and here we examine the hypothesis that this secretion contributes to a short LFNG intracellular half-life, facilitating rapid oscillations within the segmentation clock. We localize N-terminal protein sequences that control the secretory behavior of fringe proteins and find that LFNG processing is promoted by specific proprotein convertases including furin and SPC6. Mutations that alter LFNG processing increase its intracellular half-life without preventing its secretion. These mutations do not affect the specificity of LFNG function in the Notch pathway, thus regulation of protein half-life affects the duration of LFNG activity without altering its function. Finally, the embryonic expression pattern of *Spc6* suggests a role in terminating LFNG activity during somite patterning. These results have important implications for the mechanisms that contribute to the tight control of Notch signaling during vertebrate segmentation.

The Notch signaling pathway is a widely expressed, highly conserved pathway involved in numerous developmental mechanisms [1]. The central events of Notch signaling are straightforward. NOTCH receptor binding to JAGGED and/or DELTALIKE ligands, (SERRATE and DELTA in *Drosophila*) promotes the release of the intracellular receptor domain (NICD) by a series of cleavage events. NICD translocates to the nucleus and promotes transcription of target genes [2]. This pathway can be controlled spatially and temporally through modification of NOTCH receptors by *fringe* family proteins, which in vertebrates include Lunatic, Manic, and Radical fringe (*Lfng*, *Mfng* and *Rfng*) [3,4]. *Fringe* genes encode glycosyltransferases that modulate Notch signaling by modification of the NOTCH extracellular domain [5,6]. FRINGE proteins transfer *N*-acetylglucosamine to fucose on extracellular EGF repeats of NOTCH receptors, but it is not well understood how sugar addition alters the interactions between NOTCH and its ligands [7]. In vitro reconstitution of the NOTCH:ligand interactions utilizing *Drosophila* proteins finds that glycosylation of the receptor enhances its binding to DELTA, but inhibits its binding to SERRATE [8]. In mammalian systems, however, different fringe proteins have distinct effects on Notch signaling, perhaps allowing for context-dependent fine-tuning of Notch signaling (for example [9–11]).

One embryonic process that requires the modulation of Notch signaling by LFNG is vertebrate segmentation [2,12,13]. Notch signaling and *Lfng* expression play multiple roles during somitogenesis. In the posterior presomitic mesoderm (PSM) of many vertebrates, oscillatory NOTCH1 activity is regulated, at least in part, by feedback loops that involve the modulation of NOTCH1 by LFNG. Cyclic activation of NOTCH1 is important in the segmentation clock that times the process of somitogenesis. In the anterior PSM, NOTCH1 is critical for the rostral/caudal (R/C) patterning of somites, and here again LFNG may play key roles in localizing Notch signaling to the appropriate compartment [12,14].

For *Lfng* to play a role in the segmentation clock, its protein activity levels must oscillate with a short period (two hours in the mouse). During chick segmentation, LFNG protein levels, as well as *Lfng* transcript levels, oscillate with a period that matches somite formation, linking LFNG protein activity to the clock [15]. Cyclic *Lfng* expression is regulated transcriptionally [16,17], but little is known about the post-translational mechanisms that contribute to the rapid periodicity of its function in the segmentation clock. The functions of FRINGE proteins within the Notch pathway are cell autonomous [5,10,18–20], but interestingly, both *Drosophila* FRINGE and mouse LFNG protein are cleaved following a conserved dibasic site, and are secreted into the media when expressed in tissue culture cells [3]. This suggests the possibility that LFNG secretion could provide a mechanism to terminate LFNG function in the Notch pathway, facilitating the rapid oscillations of LFNG activity in the segmentation clock.

The sequence of the identified LFNG processing site (RARR in mouse) suggests that the protein may be cleaved by members of the

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subtilisin-like proprotein convertase family (SPCs) [3]. This family has nine known members that play diverse roles in the processing and maturation of many substrates including proteases, hormones and growth factors [21]. Seven of these proteins (furin/Pcsk3/SPC1, SPC2/Pcsk2/PC2, SPC3/Pcsk1/PC1/3, SPC4/Pcsk6/PACE4, SPC5/Pcsk4/PC4, SPC6/Pcsk5/PC6, and SPC7/Pcsk7) process their substrates at multi-basic sites with the motif (K/R)XX(K/R), and in many cases this protein processing is required for activation of the substrate. To better understand the post-translational regulation of LFNG activity, we examined the roles of LFNG processing by SPC convertases.

Materials and methods

LFNG mutants

AP-tagged mouse FRINGE coding sequences [3] were ligated into pcDNA3 (Invitrogen). For HA-tagged vectors the FRINGE coding sequences were transferred into a pcDNA3 vector with a C-terminal HA tag. *Rfng* or *Mfng* N-terminal sequences were amplified and RFNG aa 1–59 or MFNG aa 1–54 replaced LFNG aa 1–112 in R/LFNG and M/LFNG respectively. LFNG^{m1} (RARR to AAAA) and LFNG^{m2} (RGRR to AAAA) were created by 2 step PCR based mutagenesis (primer sequences in SI Table 1).

Alkaline phosphatase assays

4×10^4 NIH3T3 cells (grown in DMEM supplemented with 10% FBS, 50 mM glutamine) were plated in 24-well plates and co-transfected 24 h later with 800 ng of AP-fringe plasmid and 200 ng of pSVβgal (Promega) using Lipofectamine 2000 (Invitrogen). After 24 h, media was collected and cells were lysed with 100 µl Passive Lysis Buffer (Promega). 50 µl of the cellular extracts or 50 µl of heat inactivated culture was mixed with 50 µl of AP Assay Reagent A (GenHunter) to determine AP activity following the manufacturer's instructions. AP activity was calculated as (OD_{405}*54)/(Reaction time*Sample Volume) minus the background AP activity of pCDNA3 control and was normalized to β-gal activity levels as a control for transfection efficiency. 30 µl of the cell assayed by mixing with the substrate ONPG, using standard protocols. After incubation at 37 °C until a yellow color was detected, reactions were stopped by addition of 1 M Na_2CO_3, and optical density was measured at 420 nm. For each experiment the percent of AP activity in the media and in the cellular fraction were calculated.

Immunofluorescence

Cells were plated on glass cover slips, transfected as described above and fixed in 8% PFA. Coverslips were incubated with anti-AP antibody (Fitzgerald Industries, 1:100) and anti-GM130 antibody (BD Biosciences, 1:200). Secondary Alexafluor antibodies (594 anti-rabbit and 488 anti-mouse, Invitrogen) were diluted 1:1000. Cells were counterstained with Hoechst dye. Coverslips were mounted with Citifluor and examined with an Olympus 1X81 microscope. Each experiment was performed at least twice and multiple fields of cells were examined to determine intracellular localization.

NOTCH1 signaling assay

An established Notch signaling assay was utilized to assess the effects of fringe proteins on JAGGED1 induced signaling [10]. NIH3T3 cells were plated as described above and transfected with 100 ng of pBOSrNotch1 [22], 100 ng of AP-tagged fringe expression vector or empty APtag4 expression vector, 200 ng of a CBF1-luciferase reporter construct [23], and 200 ng pSVβGal for normalization of transfection efficiency. After 16 h, the cells were co-cultured for 24 h with 1.24×10^6 control L-cells or L-cells stably expressing JAGGED1 [24]. 20 µl of cell lysates were analyzed by luciferase assay (Promega). Luciferase values were normalized to βgal expression (measured as above). Notch-induced activation of CBF1 is expressed as a ratio of normalized luciferase values induced by the JAGGED-expressing cells compared to that obtained with parental L-cells.

Western blot analysis

NIH3T3 cells were plated and transfected as above with expression vectors encoding HA-tagged fringe proteins, and 6 h after transfection the media was changed to DMEM+2% FBS. Expression vectors encoding α_1PDX protease inhibitor [25], SPC1/furin, SPC4, or SPC7 [26], SPC6A or SPC6B (from D. Constam) were co-transfected as indicated. For Fig. 1A, plasmid amounts were: 350 ng LFNG, α_1PDX as indicated, and pcDNA3 to bring up total DNA to 900 ng. For Fig. 1B plasmid amounts were: 300 ng LFNG, 100 ng α_1PDX, 500 ng SPC vector as indicated and pcDNA3 to bring up total DNA to 900 ng. For Fig. 2 a total of 1000 ng of DNA was transfected, either fringe expression vector alone or equal amounts of fringe expression vector, α_1PDX and/or SPC6A expression vector. After incubating cells in DMEM+2% FBS for 24 h, 500 µl of cell media was concentrated to ~100 µl with Microcon columns (Millipore). 11.5 µl of concentrated media were mixed with 2× Laemmli loading buffer, run on a 12% polyacrylamide gel and transferred to an Immobilon membrane (Millipore). The membrane was incubated with an anti-HA antibody (HA-7, 1:1000, Sigma-Aldrich) and analyzed using the ECL System following the manufacturer's protocols (GE Healthcare).

Cycloheximide treatment

2.6×10^5 NIH3T3 cells were plated in a 6-well plate and transfected with 300 ng (LFNG, LFNG^{m1}) or 100 ng (LFNG$^{m1/2}$, R/LFNG) HA-tagged expression vectors using Lipofectamine 2000 (Invitrogen) resulting in similar, low levels of protein expression. After 6 h, transfected cells were split and 1.25×10^5 cells were plated into five wells of a 24-well plate, ensuring that all samples in the time course were transfected with equivalent efficiency. After 16 h, cells were incubated in media with 20 µg/ml cycloheximide (Sigma). Cell extracts were collected at 20-minute intervals, lysed directly in 100 µl Laemmli loading buffer, and 23 µl of each sample/time point was analyzed by Western blot

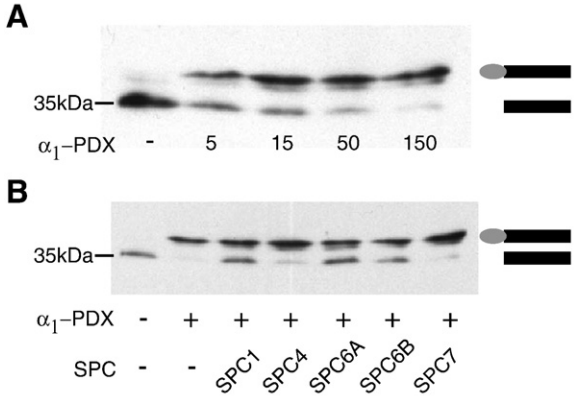

Fig. 1. LFNG is processed by SPC proconvertases. A. Media fractions from NIH3T3 cells transfected with expression vectors encoding wild-type mouse LFNG (LFNG^{wt}) and α_1PDX were analyzed by Western blot (ng of α_1PDX expression vector indicated). Protein species are diagrammed, (black box = mature LFNG, gray oval = LFNG pro region). α_1PDX expression reduces the amount of secreted mature LFNG (35 kDa) and increases the amount of full-length LFNG (43.6 kDa) suggesting a general inhibition of LFNG processing. C. NIH3T3 cells were transfected with LFNG expression vector, α_1PDX expression vector, and SPC protease expression vectors as indicated, and media fractions were analyzed by Western blot. SPC1, SPC6A, and SPC6B efficiently compete with the inhibitor and cleave LFNG, while SPC4 and SPC7 are less efficient.