Numb Is an Endocytic Protein

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Abstract. Numb is a protein that in Drosophila determines cell fate as a result of its asymmetric partitioning at mitosis. The function of Numb has been linked to its ability to bind to and to biologically antagonize Notch, a membrane receptor that also specifies cell fate. The biochemical mechanisms underlying the action of Numb, however, are still largely unknown. The wide pattern of expression of Numb suggests a general function in cellular homeostasis that could be additional to, or part of, its action in fate determination. Such a function could be endocytosis, as suggested by the interaction of Numb with Eps15, a component of the endocytic machinery.

Introduction

Drosophila Numb (dNumb)1 was originally discovered as a mutation that removed most of the sensory neurons in the developing peripheral nervous system (Uemura et al., 1989; Rhyu et al., 1994). It is now established that dNumb determines cell fate during nervous and muscle system development, as well as during Malpighian tubule development (Rhyu et al., 1994; Spana et al., 1995; Ruiz Gomez and Bate, 1997; Carmona et al., 1998; Wan et al., 2000). Asymmetric segregation of dNumb at mitosis results in its unbalanced partitioning and alternative developmental fates of daughter cells (Rhyu et al., 1994; Knoblich et al., 1995; Spana et al., 1995). Alteration of this asymmetry, by either overexpression or deletion of dNumb, leads to sibling cells adopting the same identity. Similar results were recently obtained with the avian homologue of Numb (Wakamatsu et al., 1999). There is evidence that mammalian Numb (mNumb) may exert similar functions: (a) ec-

1Abbreviations used in this paper: aa, amino acid(s); dNumb, Drosophila Numb; DPF, Asp-Pro-Phe; DLA, Asp-Leu-Ala; EH, Eps15 homology; GST, glutathione S-transferase; HA, hemagglutinin; NPF, Asn-Pro-Phe; mNumb, mammalian Numb; Tf, transferrin; TfR, Tf receptor.
act in vivo and in vitro with Eps15, a component of the endocytic machinery (Salcini et al., 1997). Eps15 has a modular structure comprising three copies of a protein–protein interaction domain, the Eps15 homology (EH) domain (Wong et al., 1995), a central coiled coil domain, and a COOH-terminal region that binds to the clathrin adaptor complex AP2 (Benmerah et al., 1995, 1996; Iannolo et al., 1997). An Asn-Pro-Phe (NPF) motif acts as the ligand for the EH domain (Salcini et al., 1997). EH-containing and EH-binding proteins establish a complex network of interactions within the cell that regulates internalization and/or trafficking processes (Santolini et al., 1999). Numb is an EH-interacting protein that binds to Eps15, and to the related protein Eps15R, through a NPF motif contained in its COOH-terminal region. This observation led to the experimental hypothesis tested in this study that Numb might itself be an endocytic protein.

Materials and Methods

Plasmids

Hemagglutinin (HA)-tagged full-length Numb, NumbL, and Numb deletion mutants (Numb/347–388 and Numb/347–564) were subcloned into pCDNA3 and pCDNA1, respectively (Invitrogen). The HA–Numb/Asp-Leu-Ala (DLA) mutant was generated by changing the sequence encoding for the Asp-Pro-Phe (DPF) motif at amino acids (aa) 566–568 to a sequence encoding for DLA by PCR-based oligonucleotide-directed mutagenesis. Glutathione S-transferase (GST) fusion proteins, containing Numb deletion mutants, were obtained by recombinant PCR of the appropriate fragment from the full-length Numb clone followed by cloning in the pGEX expression vector, in frame with the GST moiety. Sequences of the primers used are available upon request.

Protein Studies

Transfections were carried out by standard calcium phosphate protocols. Polyclonal anti-Numb antibodies were elicited against GST-Numb fusion proteins or against a peptide (encompassing aa 298–603 and 537–551 of human Numb, respectively), and affinity purified onto the same antigen used for the immunization. Other antibodies used were the anti-HA mAb 12CA5, the mAb anti–γ adaptin (A4200; Sigma-Aldrich), mAbs anti–α adaptin (A4325 for Western blotting from Sigma-Aldrich; A4320 for immunoprecipitation, AP6 for immunofluorescence and electron microscopy from Transduction Laboratories), anti-transferrin receptor (TIR) (K-20, Santa Cruz Biotechnology, Inc.), anti-EGFR (AB-5; Calbiochem), or anti-Eps15 (Fazioli et al., 1993). Immunoprecipitation and immunoblotting were performed as described previously (Fazioli et al., 1993). Purification of the GST fusion proteins onto agarose–glutathione and in vitro binding experiments were performed as described previously (Fazioli et al., 1993; Salcini et al., 1997).

For the competition experiment (see Fig. 4 D), agarose-immobilized GST–ω ear (2 μg) was incubated with 15,000 cpm of in vitro-translated Eps15 COOH-terminal region (aa 581–874) in the presence of either the purified COOH-terminal domains of Numb (aa 298–603) or Eps15 (aa 581–874), or purified BSA (Boehringer), at the indicated molar concentrations with respect to the ω ear. The purified COOH-terminal domains of Numb and Eps15, used as competitors, were produced as GST fusion proteins, cleaved from the GST moiety by thrombin digestion, and purified by fast performance liquid chromatography according to standard protocols. After 2 h incubation at 4°C, samples were washed and analyzed by SDS-PAGE, followed by autoradiography. The radioactive bands were excised and counted in a β counter.

Immunofluorescence, Electron Microscopy, and Endocytosis Assays

Indirect immunofluorescence and endocytosis assays were performed as described previously (Carbone et al., 1997).

For immunoelectron microscopy, 10% gelatin–embedded, 2.3 M sucrose–infused blocks of aldehyde-fixed A172 cells were frozen in liquid nitrogen. Ultrathin cryosections were obtained with a Reichert-Jung Ultracut E with FC4E cryoattachment and collected on copper-formvar–carbon–coated grids. Single and double immunogold localization on ultrathin cryosections were performed as described previously (Schiaffino et al., 1999). In particular, for double labeling, sections immunostained with the anti-Numb antibody, followed by 10 nm protein A–gold, were incubated with 1% glutaraldehyde in 0.1 M sodium phosphate buffer, to quench free protein A. Sections were then incubated alternatively with antibodies against AP2 (AP6, mouse), AP1 (A4200 Sigma-Aldrich), EGFR (AB-5, mouse), or TIR (K20, goat). After washing, an appropriate rabbit anti-mouse or rabbit anti–goat bridging antibody was used (Dako), followed by 15 nm protein A–gold. Control sections were incubated with an unrelated antibody or without first antibodies. To determine quenching efficiency, sections incubated with anti-Numb antibodies were quenched with 1% glutaraldehyde in 0.1 M sodium phosphate buffer, and challenged with protein A. In all control sections, no labeling was detected (data not shown). Sections were examined with a ZEISS EM 902 electron microscope.

Results and Discussion

Numb Localizes to Endocytic Organelles

Numb identifies a family of proteins, to which Numbl (also designated Numb-R and numblike) also belongs. Numb shares colinear topology and extensive sequence identity with Numb (Salcini et al., 1997; Zhong et al., 1997). Polyclonal sera directed against aa 298–603 of human Numb (hereafter Numb) cross-reacted with Numbl (Fig. 1 A). However, an antipeptide serum directed against aa 537–551 of Numb, which are not present in Numbl, specifically reacted with Numb and showed no cross-reaction with Numbl. Therefore, this serum was used in all subsequent experiments. In a panel of cell lines, Numb expression was ubiquitously detected, as shown by RNase protection assays (data not shown) and confirmed by Western blot analysis (Fig. 1 B). Both the endogenous and overexpressed Numb migrated as a tightly spaced doublet of ~70 kD (Fig. 1, A and B). Phosphatase treatment of anti-Numb immunoprecipitates yielded a single band, comigrating with the lower band of the doublet (data not shown), suggesting that the upper band is the result of phosphorylation events.

In many cell lines of nonneuronal origin, we did not observe asymmetric distribution of Numb at mitosis (data not shown). However, in all cell lines in logarithmic

<p>| Table I. Colocalization of Numb with Various Endocytic Proteins on Individual Organelles |
|-----------------------------------------------|-----------|-----------|-----------|</p>
<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
<th>A + B</th>
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<tr>
<td>Coated pits</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eps15</td>
<td>EGFR</td>
<td>10</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Eps15</td>
<td>TIR</td>
<td>1</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>Eps15</td>
<td>Numb</td>
<td>16</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Numb</td>
<td>EGFR</td>
<td>6</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Numb</td>
<td>TIR</td>
<td>3</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Numb</td>
<td>AP2</td>
<td>5</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Coated vesicles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP2</td>
<td>Numb</td>
<td>4</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Endosomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eps15</td>
<td>Numb</td>
<td>8</td>
<td>5</td>
<td>11</td>
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</table>

Double labelings were performed with the indicated antibodies (A and B). In each set of double labelings, organelles that were positive for either one of the proteins (A or B) or for both (A + B) were scored.
growth, Numb signal, as detected by indirect immunofluorescence, appeared punctate and partially concentrated in the perinuclear region, in what appeared to be the Golgi region (Fig. 1 C). Some signal was also evident at the cell periphery (Fig. 1 C). Double immunostainings revealed remarkable colocalization of Numb with adaptin (data not shown). Colocalization with adaptin in the perinuclear area was also detected (not shown).

To examine in detail the intracellular distribution of Numb, we performed immunogold labeling on ultrathin cryosections and found Numb associated to endosomes, clathrin-coated pits, and vesicles (Fig. 1 D and Table I). Immunoreactivity for Numb was also evidenced in locations compatible with the Golgi area and the TGN (Fig. 1 D). Double labelings with anti-Numb and anti–adaptin (Fig. 1 D) also supported the idea of presence of Numb in the TGN. A morphometric analysis revealed good colocalization of Numb with both EGF and Tf receptors in coated pits and with AP2 in coated pits and vesicles (Table I). Surprisingly, when double labeling for Numb and Eps15 was performed, we could not detect the presence of the two proteins in the same coated pit, despite their individual presence in different pits (Table I). In endosomes, however, Numb and Eps15 were found together on the same organelle (Fig. 1 E and Table I).

**Numb Is Redistributed upon Growth Factor Stimulation and Cotrafficks with Internalizing Receptors**

We investigated changes in the subcellular distribution of Numb. In particular, to follow Numb during the endocytic process, serum-starved cells (Starved) were treated with EGF at 4°C for 60 min (T0), followed by shifts at 37°C for 2 min (T2) and 10 min (T10). Cells were also treated with 5 nm of BSA-gold as tracer of the endocytic pathway. Two sets of double labeling were performed: (a) anti-Numb/anti-AP2 (detected with 10 and 15 nm of protein A–gold, respectively (Fig. 2, A–C, and Table II), and (b) anti-Numb/anti-EGFR (detected with 10 and 15 nm of protein A–gold, respectively (Fig. 2, D–F, and Table II).

In ST and T0, Numb localized to endocytic organelles. (A) HeLa cells transfected with HA-Numb (Numb), HA-Numbl (Numbl), or mock transfected (mock) were analyzed by immunoblotting with the antibodies indicated underneath. (B) Cellular lysates from the indicated cell lines, as well as from the mock- and HA-Numb-transfected HeLa were analyzed with the anti-Numb antibody (aa 537–551) or with the same antibody preabsorbed with the cognate peptide. (C) CV1 cells were stained with the anti-Numb antibody (aa 537–551) or with same antibody preabsorbed with the cognate peptide, as indicated. Similar results were observed in HeLa, COS-1, A172, M413, U2OS, and VA13 cells (data not shown). (D) Immunoelectron microscopy performed on exponentially growing A172 cells. In all panels, except the TGN (Numb/API) panel, staining was with the anti-Numb antibody (aa 537–551, 15 nm gold). BSA was used as a tracer of endocytosis by incubating the cells for 1 h with BSA-gold/5 nm beads before fixation. Representative examples are shown of the TGN, coated pits (CP), coated vesicles (CV), and endosomes (end).
bulk of internalizing Numb-positive coated pits and vesicles was induced, whereas Numb labeling was strongly reduced in the TGN area. At T10, a decrease in Numb-positive coated pits and vesicles was accompanied by an increase in endosome staining (Table II). These results indicate that upon internalization of the EGFR, Numb is translocated from the TGN to endocytic compartments. In these compartments, Numb appears to be cotrafficked with the internalizing receptors, first to vesicle intermediates and then to endosomes. As shown, Numb colocalizes with AP2 (Fig. 2, A–C) and EGFR (Fig. 2, D–F) at all stations of the endocytic route. Numb also appeared to be cotrafficked with the TfR, in that double staining with anti-Numb and anti-TfR revealed colocalization of the two proteins in all endocytic compartments (Fig. 2, G–I).

### Table II. Association of Numb with Endocytic Organelles

<table>
<thead>
<tr>
<th></th>
<th>TGNs</th>
<th>CPs</th>
<th>CVs</th>
<th>Endosomes</th>
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<tbody>
<tr>
<td>Growing</td>
<td>39.1 (29)</td>
<td>12.5 (54)</td>
<td>24.3 (102)</td>
<td>24.1 (35)</td>
</tr>
<tr>
<td>Starved</td>
<td>92.4 (42)</td>
<td>1.8 (29)</td>
<td>0.6 (36)</td>
<td>5.2 (27)</td>
</tr>
<tr>
<td>T0</td>
<td>93.4 (26)</td>
<td>2.2 (37)</td>
<td>0.5 (16)</td>
<td>3.9 (29)</td>
</tr>
<tr>
<td>T2</td>
<td>41.6 (32)</td>
<td>15.5 (63)</td>
<td>26.5 (105)</td>
<td>16.4 (32)</td>
</tr>
<tr>
<td>T10</td>
<td>18.5 (19)</td>
<td>6.6 (30)</td>
<td>8.5 (58)</td>
<td>66.4 (37)</td>
</tr>
</tbody>
</table>

A172 cells in logarithmic growth (Growing) were analyzed. Alternatively, A172 cells were serum starved (Starved) and then treated with EGF at 4°C for 60 min (T0), followed by shift at 37°C for 2 min (T2) or 10 min (T10). Immunoelectron microscopy was performed by staining with anti-Numb (537–551), followed by 10 nm gold protein A. For each of these experimental conditions, the total number of Numb-associated gold particles was counted, as well as the number of gold particles associated with TGNs, coated pits (CPs), coated vesicles (CVs), and endosomes. Values in the table represent the number of gold particles associated to each organelle, expressed as a percentage of the total number of gold particles for each condition of treatment. 10 cell profiles were analyzed for each condition, which resulted in at least 100 gold particles counted for each condition. The number of CPs, CVs, and endosomes counted in each cell profile is given in parentheses to serve as a reference. A fixed area corresponding to the small fluorescent screen of the microscope at 12,000× was used to divide TGNs in portions; the number in parentheses indicates the total number of such areas scored.

### Numb Binds Specifically and Directly to the Appendage of α Adaptin

Numb could be recruited to endocytic organelles through interaction with Eps15. However, the lack of colocalization of Numb and Eps15 in the same coated pit posed the question as to whether Numb interacts with other component(s) of the endocytic machinery.

A GST fusion protein containing aa 63–603 of Numb could efficiently bind to α adaptin from cellular lysates, but not to the γ subunit of the AP1 complex (Fig. 3 A). The binding was mediated through the appendage of α adaptin (α ear) (Fig. 3 B), and was direct, since a GST–α ear was able to bind to the COOH-terminal domain of Numb, recombinantly produced and purified (Fig. 3 C). Finally, endogenous Numb and α adaptin could be readily coimmunoprecipitated from cell lysates (Fig. 3 D).

To map the region of Numb responsible for interaction with α adaptin, we tested fragments of Numb engineered as GST fusion proteins (Fig. 4 A). A region between position 564 and 588 contained the determinants necessary for...
the interaction (Fig. 4, A and B). This region contained a DPF motif (aa positions 566–568), which has been recently shown to constitute a ligand for the α ear and is present in several proteins known to bind to the appendage domain of α adaptin (Owen et al., 1999). We engineered an HA-tagged mutant of Numb, in which the sequence encoding for DPF was mutagenized to one encoding DLA. This mutant could not coimmunoprecipitate α adaptin (Fig. 4 C), indicating that the DPF motif in position 566–568 of Numb is responsible for its interaction with α adaptin. Of interest, the DPF motif identified is conserved in evolution, being also present in dNumb (aa 503–505).

The existence of a single binding site on α ear, as determined by the resolution of its structure (Owen et al., 1999), predicts competition between Numb and other α ear–associated proteins. We tested this prediction experimentally, by competing the binding of Eps15 to α ear with increasing concentrations of Numb (Fig. 4 D). The COOH-terminal region of Numb was indeed able to compete the interaction between the COOH-terminal domain of Eps15 (which contains all the determinants necessary and sufficient for binding to α adaptin) and a GST–α ear fusion protein. However, it did so with lower efficiency than the COOH-terminal portion of Eps15 itself, suggesting that, in vivo, Eps15 binds to AP2 with higher affinity than with Numb (Fig. 4 D).

**Fragments of Numb Act as Dominant Negatives on Endocytosis**

To test whether perturbation of Numb function might interfere with the endocytic process, we overexpressed, in CV1 cells, COOH-terminal fragments of Numb, Numb/347–588, and Numb/347–564 and monitored the uptake of fluorochrome-conjugated EGF and Tf. Both COOH-terminal fragments inhibited internalization of EGF and Tf (Fig. 5). Control GFP did not affect internalization (Fig. 5). Of note, the Numb/347–564 mutant did not retain binding sites for either AP2 (DPF) or Eps15 (NPF), thus ex-
cluding that in vivo titration of these two known endocytic components was responsible for the effect. These data functionally establish Numb as a component of the endocytic machinery. They further identify an unexpected region of Numb (aa 347–564), which does not bind to either AP2 or Eps15 as an important determinant for Numb function in endocytosis.

**Does Numb Act at Multiple Steps in Endocytosis?**

A role for Numb at various steps of endocytosis is suggested by its multiple, and topographically distinct interactions with the endocytic machinery. Numb binds to α adaptin and Eps15, albeit not simultaneously, at least in coated pits, where Numb and Eps15 do not colocalize. Eps15 binds to α adaptin with higher affinity than Numb, suggesting that in pits, Eps15 must be first disengaged from AP2 for Numb to bind, consistent with findings that the Eps15–AP2 is progressively disrupted upon coat assembly (Cupers et al., 1998). Thus, Numb and Eps15 might participate to the assembly of pits in a temporal hierarchy. Conversely, a Numb–Eps15 complex might function at the endosome level. We note that, after its release from coated pits, Eps15 is retargeted to endosomes (Torrisi et al., 1999), playing a yet uncharacterized role that requires, however, its EH domains (Torrisi et al., 1999). Thus, EH-mediated interactions of Eps15 with target molecules are important in endosomes, where Eps15 and Numb colocalize, raising the possibility that Numb might be an effector of Eps15 in this location. Finally, a fragment of Numb, which cannot bind to either AP2 or Eps15, acts as a dominant negative on endocytosis, suggesting interactions with other endocytic proteins.

The participation of Numb to endocytosis asks whether the role of Numb in development is linked to this process. Although there is evidence (Seugnet et al., 1997; Parks et al., 2000) that Notch signaling might require its endocytosis, it is conceivable that upon prolonged stimulation, and by analogy to most signaling receptors, endocytosis of Notch would counteract its signaling. Thus, in principle, the antagonistic effect of Numb on Notch could be explained, at least in part, by Numb-dependent Notch internalization with ensuing attenuation of Notch signaling. Although this speculative scenario awaits experimental testing, our results, when viewed in the context of Numb action on development, raise the possibility that cell fate determination could be due, at least in part, to asymmetric partitioning of endocytic machinery at mitosis.

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**Figure 5.** Numb inhibits clathrin-mediated internalization. CV1 cells were transfected with pCDNA3 expression vectors encoding either HA-Numb/347–588 (A and D) or HA-Numb/347–564 (B and E), or GFP as a control (C and F). The cells were then incubated with either rhodamine-conjugated EGF (A–C) or rhodamine-conjugated Tf (D–F). Transfected cells were detected with either an anti-Numb antibody (A, B, D, and E) or by direct GFP fluorescence (C and F), and are marked with asterisks. Internalized EGF or Tf is visible in red. More than 50% of the cells transfected with Numb deletion mutants presented a complete block in EGF and Tf uptake in three independent experiments. Transfection of full-length Numb did not alter the initial rate of endocytosis of both EGFR and TIR (data not shown).
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