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Motifs that mediate dendritic targeting in hippocampal neurons: A comparison with basolateral targeting signals

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One model for dendritic protein sorting in neurons is based on parallels with basolateral targeting in Madin–Darby Canine Kidney (MDCK) epithelial cells. The goal of this study was to further evaluate this model by analyzing the neuronal targeting of several proteins that contain well-defined basolateral sorting motifs. When we expressed FcRγII-B2 and CD44, two basolateral markers whose sorting depends on dihydrophobic motifs, they were unpolarized in hippocampal neurons. We also assessed the localization of the Epidermal Growth Factor Receptor (EGFR), a basolateral protein whose sorting signal contains a proline-rich motif and two dihydrophobic motifs. EGFR was restricted to the dendrites in neurons and relied on the same sorting signal for proper targeting. These results show that the dendritic sorting machinery in neurons does not recognize dihydrophobic-based basolateral sorting signals. In contrast, the sorting signal present in EGFR directs both basolateral and dendritic targeting and defines a novel dendritic targeting motif.

Introduction

Neurons are polarized into two functionally and biochemically distinct domains: somatodendritic and axonal. Proper neuronal function depends on the accurate sorting and targeting of membrane proteins, such as neurotransmitter receptors and cell adhesion molecules, to these domains (Craig and Banker, 1994). Like neurons, epithelial cells maintain two different domains within their plasma membrane: basolateral and apical. Based on a comparison of the targeting of viral proteins in Madin–Darby Canine Kidney (MDCK) epithelial cells and hippocampal neurons, Dotti and colleagues hypothesized that neurons and MDCK cells use common mechanisms for sorting membrane proteins (Dotti and Simons, 1990; see also Bradke and Dotti, 1998; Craig and Banker, 1994). In their model, the basolateral domain is equivalent to the somatodendritic domain, the apical domain to the axon. In support of this hypothesis, several basolateral proteins are targeted to dendrites when expressed in neurons. In contrast, the epithelial/neuronal parallel does not hold for apical/axonal sorting; apical proteins investigated thus far are not restricted to the axon in cultured hippocampal neurons (Jareb and Banker, 1998). Although further studies have led to revisions of the original hypothesis, comparison of polarity in MDCK cells and neurons continues to provide a useful framework for the study of polarized protein trafficking (Horton and Ehlers, 2003; Muth and Caplan, 2003; Ponnambalam and Baldwin, 2003; Winckler and Mellman, 1999).

Further support for the parallel between dendritic and basolateral targeting derives from more detailed studies of the mechanism involved. For several proteins, it has been shown that the same stretch of cytoplasmic amino acid residues mediates targeting to both somatodendritic and basolateral domains (Mostov et al., 2000). For example, in both cell types, the targeting of the Low-Density Lipoprotein Receptor (LDLR) requires a tyrosine-based sorting signal (Matter et al., 1994); targeting of the transferrin receptor (TfR) also depends on overlapping sorting signals (Odorizzi and Trowbridge, 1997; West et al., 1997). In epithelia, basolateral sorting depends on the interaction between these cytoplasmic sorting signals and specific adaptor subunits that govern cargo selection into transport vesicles. For TfR and LDLR, proper targeting relies on the presence of μ1B, a subunit of the AP-1 adaptor complex (Gan et al., 2002). This adaptor subunit is expressed in MDCK cells, but not in neurons, indicating that a different adaptor protein must mediate targeting of these proteins in neurons (Ohno et al., 1999). In addition to μ1B, the adaptor complex AP-4 is also known to function in basolateral sorting (Simmen et al., 2002). It is possible that this or other as yet unidentified adaptor molecules mediate dendritic sorting.

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The targeting of another class of basolateral proteins, which includes the macrophage Fc receptor γII-B2 (FcRγII-B2) and the Epidermal Growth Factor Receptor (EGFR), does not depend on μ1B (Ang et al., 2003; Mullin and McGinn, 1987). We were interested in knowing if the basolateral targeting signals in these proteins are also recognized by the dendritic sorting machinery. FcRγII-B2 relies on a dihydrophobic amino acid motif for basolateral sorting. Point mutations of the Leu–Leu sequence in the cytoplasmic tail of FcRγII-B2 abolish basolateral targeting in MDCK cells as assayed by confocal microscopy and cell-surface biotinylation (Sheikh and Isacke, 1996). Another basolateral protein that utilizes a dihydrophobic motif for its targeting is the hyaluronate receptor CD44, which relies on a Leu–Val sequence in its cytoplasmic tail (Sheikh and Isacke, 1996). The EGFR uses a different, though partially related, basolateral sorting signal consisting of a dominant, proline-based motif and two weaker dihydrophobic motifs (He et al., 2002). In the current study, we determined whether the basolateral targeting motifs present in these proteins act as dendritic sorting signals in cultured hippocampal neurons. When cDNAs encoding FcRγII-B2 and CD44 were expressed, they were unpolarized on the neuronal plasma membrane. In contrast, EGFR was restricted to the somatodendritic surface. Furthermore, the same mutations that disrupt EGFR sorting in epithelial cells caused its mistargeting in neurons. These results expand upon previous studies of the parallel between dendritic and basolateral sorting, showing that only a subset of basolateral sorting signals are recognized as dendritic sorting signals in neurons.

Results

CD44 and the FcRγII-B2 receptor are unpolarized when expressed in hippocampal neurons

CD44 and FcRγII-B2 are targeted to the basolateral domain in MDCK cells based on well-defined dihydrophobic motifs in their cytoplasmic tails (Hunziker and Fumey, 1994; Sheikh and Isacke, 1996). We sought to determine whether these proteins were dendritic when expressed in cultured hippocampal neurons.

To determine the cell surface distribution of CD44 and FcRγII-B2, we expressed each marker in hippocampal neurons for 18–24 h then immunostained living cells using species-specific antibodies directed against extracellular epitopes. We coexpressed soluble eGFP along with each membrane protein in order to label the entire axonal and dendritic arbor of the cell. Comparison with the distribution of this soluble marker showed that FcRγII-B2 (Figs. 1D–F) and CD44 (Figs. 1G–I) were uniformly distributed on the plasma membrane throughout both axons and dendrites. A comparable distribution was observed after expression for 5.5 h, the earliest time when the proteins could be detected on the cell surface (shown for FcRγII-B2 in Supplementary Figs. 1A, B). Intracellular labeling (revealed by fixation and permeablization before immunostaining) was associated with the Golgi complex as well as many tubulovesicular profiles, which were found throughout the neurites (Supplementary Figs. 1C–E). The latter likely correspond to the carriers that deliver these proteins to the cell surface, as revealed in previous, live-cell imaging studies (Burack et al., 2000; Kreitzer et al., 2003; Lippincott-Schwartz et al., 2000). The unpolarized surface distribution of CD44 and FcRγII-B2 was distinctly different from the localization of TfR, a basolateral protein that is polarized to the somatodendritic domain (Figs. 1A–C). To quantify the polarity of these proteins, we measured the average fluorescence intensity in dendrites and axons, using the method described by Sampo et al. (2003). The ratio of fluorescence in dendrites compared to axon (the D:A ratio) was 1.9:1 for CD44 and 1.6:1 for the FcRγII-B2 (Table 1). These values are comparable to those of unpolarized proteins assessed using this approach (Cheng et al., 2002; Sampo et al., 2003). When we quantified the polarity of TfR, whose dendritic targeting has been studied extensively (Burack et al., 2000; West et al., 1997), we found that its D:A ratio was 7.7:1 (Table 1), significantly different from the D:A ratio of FcRγII-B2 and CD44. Other dendritically polarized proteins show D:A ratios similar to that of TfR (Cheng et al., 2002; Rivera et al., 2003).

The EGF receptor is targeted to the somatodendritic domain

To determine the cell surface distribution of the EGFR in neurons, we expressed human EGFR for 18–24 h and performed live-cell immunostaining using a species-specific monoclonal antibody directed against an extracellular epitope. Cell surface fluorescence was polarized to the somatodendritic domain (Figs. 2A, B). Quantification of cell-surface fluorescence revealed that EGFR was polarized to the dendrites, with a D:A ratio of 6.6:1 (Table 1). The dendritic polarity of EGFR was somewhat reduced when expression was high, suggesting that the cellular machinery responsible for its sorting may be saturable. In cells expressing lower levels of EGFR, the D:A ratio was slightly higher, about 8:1. We did not observe a relationship between overexpression and polarity with any of the other constructs examined.

In MDCK cells, a 22-amino acid region (Lys652–Ala674) of the cytoplasmic juxtamembrane domain targets EGFR to the basolateral membrane (Hobert et al., 1997). Truncation of the entire cytoplasmic tail results in its mislocalization to the apical domain, while truncation just beyond the critical juxtamembrane region has no affect on its polarity (Fig. 3; He et al., 2002). We observed parallel results when these constructs were expressed in hippocampal neurons. When the cytoplasmic domain of EGFR was truncated at the end of the 22-amino acid sorting region (P675STOP), the protein was still accurately sorted to the dendrites. Truncation of the entire cytoplasmic tail (K652STOP) resulted in a significant loss of polarity. The D:A ratio was reduced from 7.2 to 3.2, a value slightly greater than was observed for unpolarized proteins. These data show that the same 22-amino acid domain that governs the basolateral targeting of EGFR also contains information necessary for its dendritic targeting.

Within the 22 amino acid juxtamembrane domain, He et al. (2002) identified several residues important for basolateral localization, including two dihydrophobic motifs and a novel, proline-dependent motif (Fig. 3). Mutating both prolines markedly reduced its basolateral polarization, whereas mutating both dihydrophobic motifs had only a slight effect. Mutating all six residues simultaneously reduced its polarity even further.

Expression of these truncation/substitution constructs in neurons gave parallel results. Mutation of both dihydrophobic motifs (P675STOP-LL, LV-4xA) resulted in a small, but not significant reduction in the dendritic polarity of EGFR (D:A ratio, 5.8:1, Figs. 4A, B). Mutation of both proline residues (P675STOP-PxxP-2xA) resulted in a greater reduction in polarity (D:A ratio, 4.8:1). Mutation of both dihydrophobic motifs and both proline residues (P675STOP-LL, LV, PxxP-6xA) decreased its polarity to the same

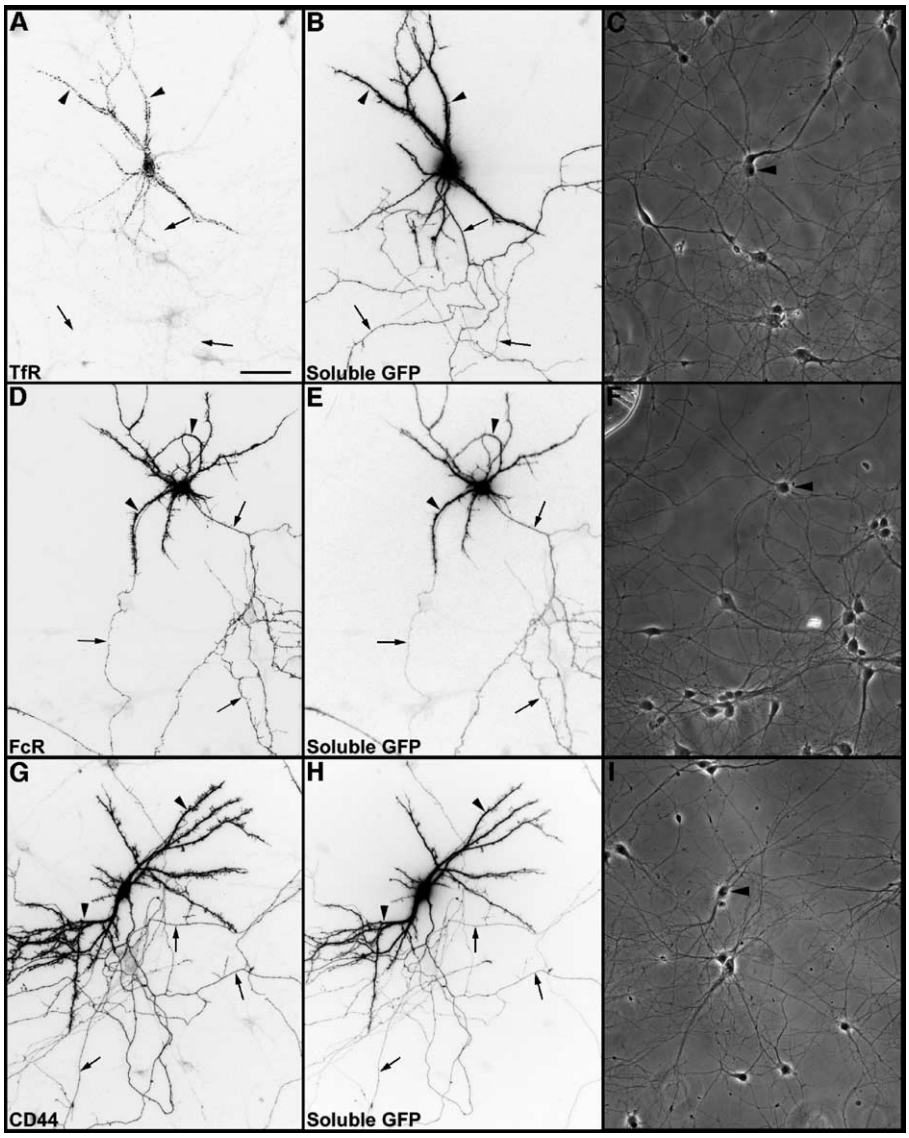

Fig. 1. The basolateral proteins CD44 and FcRγII-B2 are unpolarized when expressed in rat hippocampal neurons. Cell surface immunostaining of human TfR (A) was highly polarized to the dendrites. The axon and dendrites were identified by coexpression of soluble GFP, which fills the entire cell (B). In contrast, cell surface immunostaining of CD44 (D) and FcRγII-B2 (G) extended throughout both the dendrites and axons (compare with soluble GFP images–E, H). Phase images are shown with an arrowhead marking the cell body of the transfected cell (C, F, I). Cells from 7–9 day old cultures were doubly transfected with soluble GFP and the indicated protein then allowed to express the constructs for 18–24 h. Arrows show axons, while arrowheads denote dendrites. The contrast was inverted in all fluorescent images to enhance the visibility of thin axonal processes. Scale bar, 50 μm.

extent as truncating the entire cytoplasmic domain (D:A ratio, 3.5:1; Figs. 4C, D).

Discussion

In order to test whether multiple basolateral sorting signals are recognized by neurons, we expressed several markers with well-defined basolateral targeting signals in cultured hippocampal neurons and assayed their polarity on the cell surface. Our results show that the dihydrophobic motifs present in FcRγII-B2 and CD44 do not mediate dendritic targeting. In contrast, we found that EGFR is polarized to the somatodendritic domain in neurons and relies on the same signal that mediates its basolateral targeting in epithelial cells.

The role of dileucine targeting motifs

Two types of dihydrophobic trafficking signals have been particularly well characterized. Motifs conforming to (DE)xxxL(LI) bind to subunits of AP-1, AP-2, and AP-3, mediating endocytosis and lysosomal targeting, while DxxLL binds to the VHS domain of another class of sorting adaptors, the GGAs (Bonifacino and Traub, 2003). Basolateral dileucine motifs typically do not conform to either of these sequences. CD44 contains both an upstream and a downstream acidic residue, but neither are important for basolateral targeting (Sheikh and Isacke, 1996). FcRγII-B2 and E-Cadherin both contain an acidic residue downstream of the dileucine motif, but again this residue is not involved in targeting (Hunziker and Mellman, 1989; Miranda et al., 2001). We found that the dihydrophobic motifs in FcRγII-B2 and CD44 do not mediate

Table 1
Quantification of polarity of basolateral proteins expressed in hippocampal neurons

Protein	*n*	Polarity (dendrite:axon ratio)
TfR	8	7.7 ± 2.1
CD44	11	1.9 ± 0.56*
FcRγII-B2	15	1.6 ± 0.55*
EGFR	33	6.6 ± 2.8

Values are means ± SD.
Ideally, quantification of an unpolarized protein's distribution should yield a 1:1 ratio of fluorescence in the dendrites versus axon; however, the dendrites are five to ten times thicker than the axon, so it is likely that a greater contribution of out of focus fluorescence in the dendrites results in a dendrite:axon ratio slightly greater than one.
* Significantly different than TfR ($p < 0.05$).

dendritic targeting in neurons. Another dendritic protein, the EGFR-like protein DNER, also contains a dihydrophobic motif in its cytoplasmic tail, but this signal is not required for its dendritic targeting (Eiraku et al., 2002).

There are also examples where dihydrophobic motifs play a role in neuronal membrane trafficking. Garrido et al. (2001) have investigated the role of leucine residues in the axonal targeting of a construct consisting of the C-terminal of the Na^{2+} channel linked to a reporter protein. They show that the axonal localization of this construct depends on its selective endocytosis from the dendritic membrane, which in turn requires a dileucine motif in the cytoplasmic tail. Rivera et al. (2003) demonstrated that the dendritic targeting of the potassium channel Kv4.2 is disrupted by mutations of its cytoplasmic dileucine motif as well as of surrounding amino acid residues. In neurons, as in other cell types, it is likely that dihydrophobic motifs play multiple roles in protein trafficking, which depend in part on position and on the context of surrounding amino acid residues (Gu et al., 2001; Rohrer et al., 1996).

A novel dendritic targeting signal in EGFR

EGFR contains a short region in its cytoplasmic domain that is important for its basolateral targeting in epithelia. Within this region

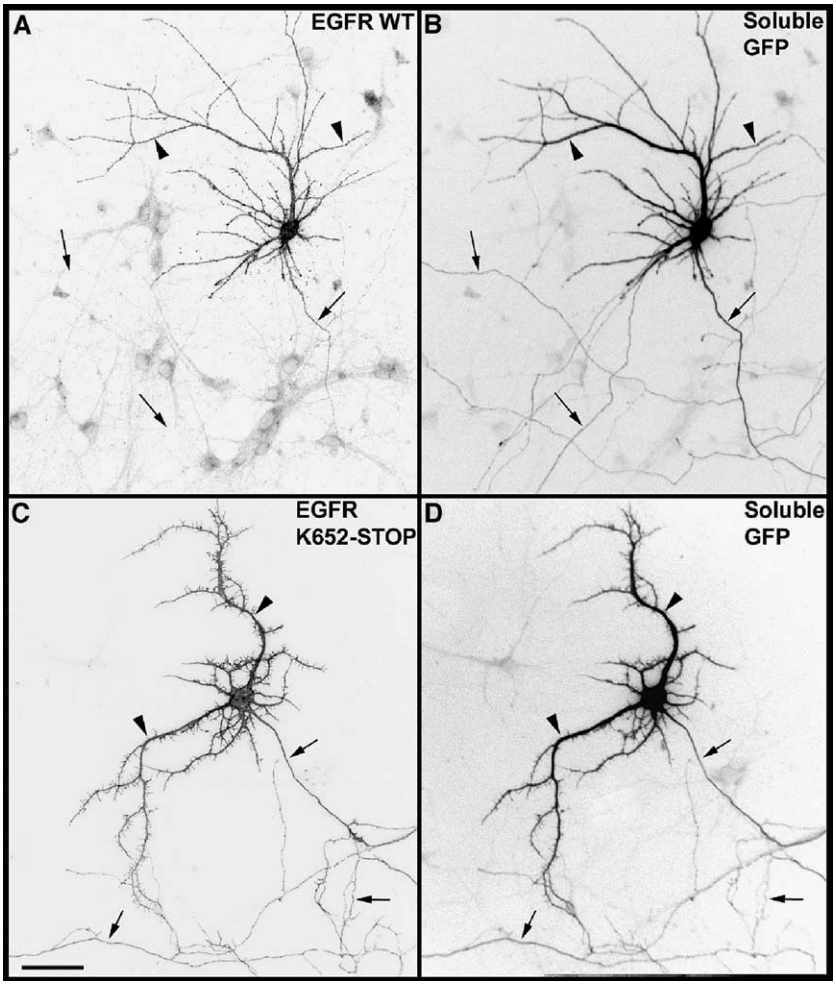

Fig. 2. Cell surface immunostaining of full-length EGFR and truncated EGFR. Wild-type EGFR was polarized to the dendritic cell surface (A). Faint staining extended into the very proximal portion of the axon. Soluble GFP labeled the entire cell, including the axon and dendrites (B). Truncation of the entire cytoplasmic domain of EGFR (EGFR K652-STOP) resulted in a marked reduction in polarity (C, D). Hippocampal neurons from 7–9 day old cultures were cotransfected with EGFR and soluble GFP, then allowed to express the constructs for 18–24 h. Arrows show axons, while arrowheads denote dendrites. Scale bar, 50 μm.

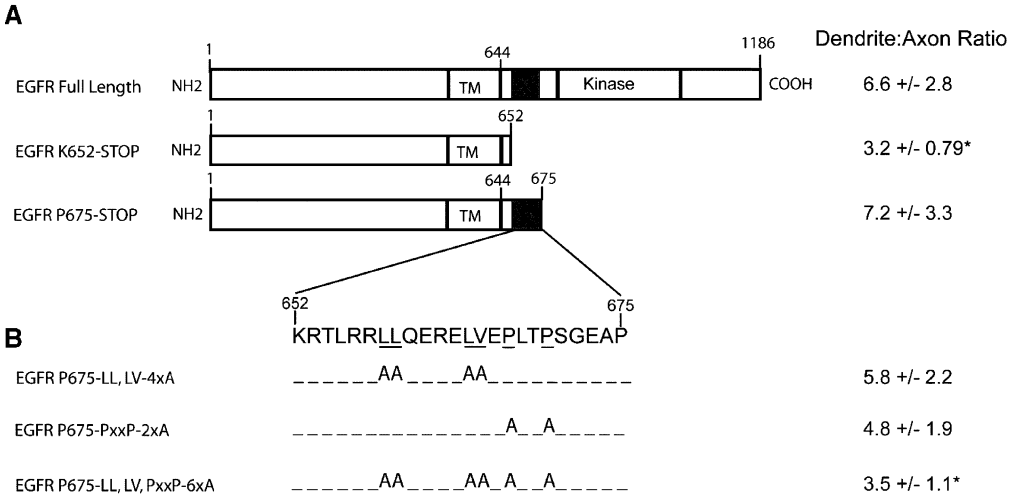

Fig. 3. Quantification of the polarity of EGFR constructs expressed in hippocampal neurons in culture. (A) Schematic drawings showing the domain structure wild-type EGFR as well as the cytoplasmic truncations, K652-STOP and P675-STOP. The area in gray shows the region necessary for basolateral localization of EGFR in MDCK cells. (B) Point mutations introduced into the P675-STOP truncation (at the positions underlined). Quantification of the polarity (dendrite:axon ratio) is shown to the right of each construct. Values are means ± standard deviations. * Significantly different from wild-type EGFR, $p < 0.05$.

lie specific amino acid motifs that are necessary for proper targeting: a dominant proline-based motif and two less important dihydrophobic motifs. We found that these motifs are also important for proper dendritic targeting. No other proline-rich dendritic targeting motifs have been described, though at least one other basolateral protein contains a similar motif within its basolateral sorting signal (Odorizzi and Trowbridge, 1997). The sorting machinery that binds to this novel targeting signal is not known; however, this PxxP motif conforms to a type I SH3-binding domain, which mediates protein–protein interactions important for signal transduction, synaptic vesicle endocytosis, and lysosomal sorting (Blott et al., 2001; Kay et al., 2000; McPherson, 1999).

When we expressed a near complete C-terminal truncation of EGFR (K652STOP), the protein was still slightly more dendritic

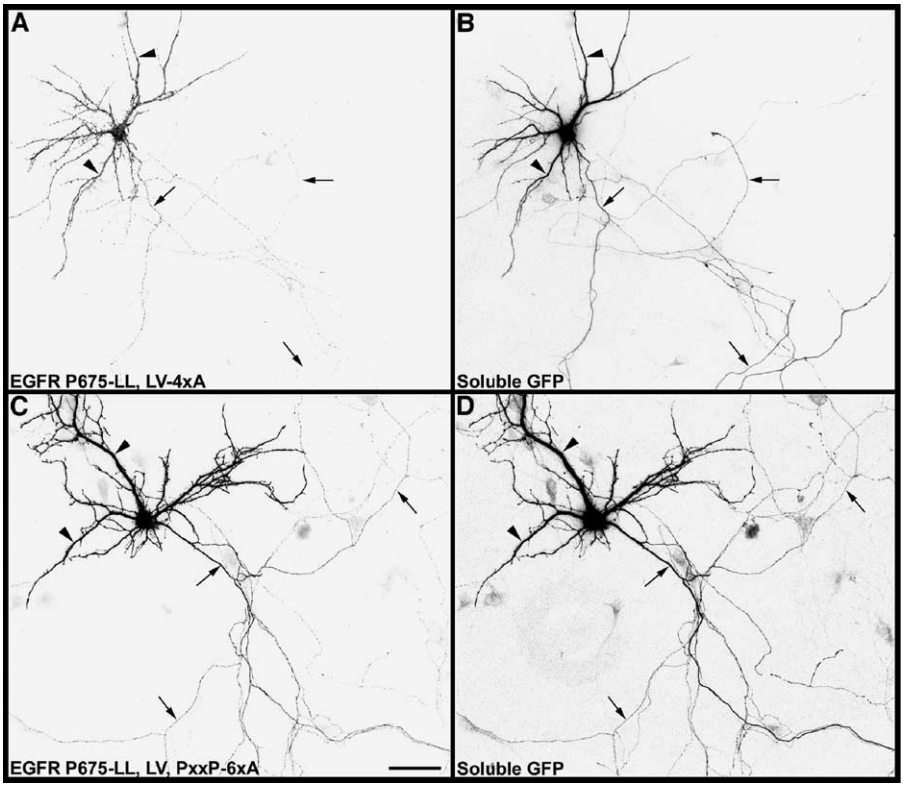

Fig. 4. Cell surface distribution of EGFR sorting signal mutants. Mutation of both dihydrophobic motifs in the cytoplasmic juxtamembrane domain of the truncated EGFR (EGFR P675-LL, LV-4xA) had little effect on the protein's dendritic distribution (A, B). Mutation of the proline-rich motif together with both dihydrophobic motifs (EGFR P675-LL, LV, PxxP-6xA) led to a significant reduction in polarity (C, D). Cells from 7–9 day old cultures were cotransfected with mutated EGFR and soluble GFP then analyzed after expression for 18–24 h. Arrows denote axons, arrowheads show dendrites. Scale bar, 50 μm.

than unpolarized proteins. It is possible, though unlikely, that the few amino acids remaining in the cytoplasmic domain contribute to dendritic targeting. Alternatively, the expressed constructs may have formed dimers with endogenously expressed copies of EGFR (Tucker et al., 1993). If this were the case, wild-type copies of EGFR, with intact dendritic targeting signals, could pull mutant copies into the proper dendritic sorting pathway, leading to partial dendritic polarity of overexpressed mutants. We have observed a similar phenomenon in the studies of the dendritic targeting of the Low-Density Lipoprotein Receptor (LDLR). When LDLR was coexpressed with a sorting signal mutant of LDLR, the wild-type protein was not completely excluded from the axon, and the mutant protein was slightly polarized to dendrites (unpub. obs.).

Conclusion

It is well documented that both dendritic and basolateral proteins rely on short amino acid sequences within their cytoplasmic tails for polarized sorting. Do the same motifs that mediate basolateral sorting also mediate dendritic sorting? In some cases, they clearly do not. As shown in this study, the basolateral proteins FcRγII-B2 and CD44 are unpolarized in neurons. In addition, basolateral proteins which utilize a different class of sorting motifs, such as TrKB, are also unpolarized when expressed in neurons (Kryl et al., 1999). A particularly striking case of disparate targeting is illustrated by the sorting of the excitatory amino acid transporter EAAT3. This protein, which is expressed endogenously in both hippocampal neurons and epithelial cells, relies on the same short stretch of amino acids for targeting to the dendrites in neurons, but to the apical domain in epithelia (Cheng et al., 2002).

Other proteins, such as TfR and LDLR, do use similar motifs for dendritic and basolateral targeting. Even for these proteins, however, there are subtle differences. For TfR, residues important for basolateral targeting are slightly distal to those important for dendritic polarity (Odorizzi and Trowbridge, 1997; West et al., 1997). In LDLR, a naturally occurring point mutation that causes mistargeting in epithelia (Koivisto et al., 2001) does not disrupt dendritic targeting (unpub. obs.). Adding a C-terminal GFP tag does not disrupt basolateral targeting of LDLR in MDCK cells (Kreitzer et al., 2003), but a nearly identical construct is unpolarized when expressed in neurons (unpub. obs). Subtle differences were also observed in the case of EGFR. For example, alanine substitution of the dihydrophobic motifs appears to have a greater effect on dendritic sorting than on basolateral sorting.

What could account for the differences between dendritic and basolateral targeting? In the case of proteins that are dendritic in neurons but not basolateral in epithelia, or vice versa, an obvious possibility is that the adaptors that recognize their sorting motifs are expressed in only one of the two cell types. Alternatively, the same adaptors may be expressed in both cell types, but downstream events may direct the proteins to different domains. Subtle differences between basolateral and dendritic sorting signals could be explained by differences in the regulation of adaptors. For example, phosphorylation could modify the interaction between sorting motifs and adaptors (Dietrich et al., 1994; von Essen et al., 2002). In addition, adaptor-modulating proteins may also be differentially expressed (Hinners et al., 2003; Rapoport et al., 1997; Shang et al., 2004). A clearer picture of the molecular details of polarized targeting in neurons will depend on further characterization of such sorting signal binding partners.

Experimental methods

Antibodies and expression vectors

We thank the following people for generously providing these reagents: Dr. I. Stamenkovic, Harvard Medical School, human CD44 cDNA; Dr. I. Mellman, Yale University, mouse FcRII-B2 cDNA; Dr. John Adelman, Vollum Institute, pJPA expression vector; Dr. C. Enns, Oregon Health and Science University, pCB6-human TfR. Golgi-CFP and eGFP were from Clontech (Palo Alto, CA). The cDNAs encoding eGFP, human CD44, and mouse FcRγII-B2 were subcloned into the pJPA expression vector. The following human EGFR cDNAs are previously described by Hobert et al. (1997) and He et al. (2002): pCB6 + EGFRWT, pCB6 + EGFR C′652STOP, pCB6 + EGFR C′674, pCB6 + EGFR C′675STOP-LL, LV-4xA, pCB6 + EGFR C′675STOP-PxxP-2xA, pCB6 + EGFR C′675STOP-LL, LV, PxxP-6xA. Monoclonal antibodies specific for human CD44 (1:100) and human EGFR (1:200) were from Santa Cruz Biotechnology (Santa Cruz, CA), mouse FcRγII-B2 (1:100) antibodies were from Pharmingen (San Diego, CA), and antibodies against human TfR (1:100) (B3/25) were from Roche (Basel, Switzerland). Working dilutions for immunocytochemistry are given in parentheses.

Hippocampal cell culture and expression of transgenes

Primary cultures of dissociated neurons from E18 embryonic rat hippocampi were prepared essentially as described (Banker and Goslin, 1998). Cells 6–8 days in vitro were transfected with the transmembrane marker protein of interest along with soluble enhanced green fluorescent protein (eGFP) using Effectene (Qiagen, Valencia, CA) or Lipofectamine2000 (Invitrogen, Carlsbad, CA). Cells were allowed to express for 4–48 h at 37°C under a controlled atmosphere containing 5% CO_2. To detect expressed proteins on the cell surface, living neurons were incubated in primary antibody diluted to the appropriate concentration in culture medium for 7–10 min at 37°C. Coverslips were then rinsed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde/4% sucrose in PBS. Cells were incubated in 0.50% fish skin gelatin (Sigma-Aldrich, St. Louis, MO) in PBS for 1 h at 37°C to block nonspecific antibody-binding sites. Coverslips were then incubated either in biotinylated secondary antibody for 1 h at 37°C followed by Cy3-conjugated streptavidin or directly in cy3 conjugated secondary antibody for 1 h at 37°C (Jackson Immunoresearch Laboratories, West Grove, PA). For immunostaining of total protein (surface and intracellular), cells were fixed as above, permeablized with Triton-X-100 (.25%), then immunostained.

Microscopy and quantitative measurements of polarity

Images of immunofluorescently labeled cells were acquired using a Princeton Instruments Micromax cooled CCD camera (Photometrics, Tuscon, AZ) controlled by Metamorph Software (Universal Imaging, Downingtown, PA). Low magnification images were taken with a 16× 0.5 N.A. Plan Fluotar objective (Leica Microsystems AG, Wetzlar, Germany). Exposure time was adjusted so that the maximum pixel value did not reach saturation. Only cells whose labeled processes did not overlap with other labeled cells were selected for quantification.

All image processing was done with Metamorph software (Universal Imaging, Downingtown, PA). Two different corrections were performed on the raw image prior to analysis of polarity. First, a shading correction was applied (based on an image of a uniformly fluorescent slide) to compensate for the uneven illumination. Next, an average background fluorescence value was determined from several regions containing unlabeled neurites then subtracted from the whole image. In order to quantify the fluorescence in axons and dendrites, several 1-pixel wide 100 μm lines were drawn on randomly selected portions of the axon and the dendrites (6–10 lines each) using the soluble GFP signal as a guide, then these regions were transferred to the live-cell immunostain image and the average fluorescence for each region was calculated. The average intensities from axons and dendrites of each cell were transferred to an Excel (Microsoft, Redmond, WA) spreadsheet. The dendrite:axon ratios for each construct were calculated from minimally 8 cells from at least two different cultures. Statistical significance was assessed using the Newman–Keuls multiple comparisons test for independent groups.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2005.02.008.

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