Ephrin signaling: One raft to rule them all? One raft to sort them? One raft to spread their call and in signaling bind them?

Laura R. Gauthier, Stephen M. Robbins*

Departments of Oncology and Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta, Canada T2N-4N1

Abstract

The Eph receptor tyrosine kinases (RTK) and their membrane-bound ligands, the ephrins, mediate cell-contact-dependent signaling events that control multiple aspects of metazoan embryonic development. The ephrins and their receptors regulate cell movement that is essential for forming and stabilizing the spatial organization of tissues and cell types. This includes the guidance of migrating cells or neuronal growth cones to specific targets. Although the biological responses mediated by the ephrin-Eph system were thought to be imparted by the Eph receptor via ‘classical’ RTK signaling pathways, there is now accumulating evidence that the ephrins are not merely ligands but have biological activity independent of the kinase activity of their cognate Eph receptor. This activity is commonly referred to as ‘reverse’ or ‘bi-directional’ signaling. Furthermore, ephrin-mediated signaling is restricted to specific membrane microdomains known as ‘lipid rafts’, which we believe imparts specificity to the extracellular signal. This review highlights the current data to support a role for lipid rafts in regulating aspects of ephrin-mediated signaling.

© 2003 Elsevier Inc. All rights reserved.

Keywords: Ephrins; Lipid rafts; Signal transduction

Eph receptors and their membrane-tethered ligands: a bi-directional signaling hypothesis

The Eph family of RTKs along with their corresponding ligands, now known collectively as the ephrins, provide cues to guide the migration of cells and growth cones during embryonic development as well as serving other distinct roles in vascular assembly and angiogenesis (for reviews see (Flanagan and Vanderhaeghen, 1998; Frisen et al., 1999; Holder and Klein, 1999; O’Leary and Wilkinson, 1999;...
Yancopoulos et al., 1998). In addition, their complementary and mutually exclusive expression patterns suggest an involvement in the formation of spatial boundaries and tissue morphogenesis during embryogenesis (Flenniken et al., 1996; Friedman and O'Leary, 1996; Gale et al., 1996). Eph receptors have recently been classified into two subfamilies, EphA and EphB, based on their sequence similarity and binding affinities for ligands that are tethered to the cell surface by either a glycosyl-phosphatidylinositol (GPI)-anchor (ephrinA) or by a single transmembrane domain (ephrinB) (Fig. 1) (Gale et al., 1996).

Many of the ephrins and Eph receptors have been shown to be expressed in the developing nervous system, where they participate in axon guidance and patterning of neuronal connections including the formation of topographic maps in the visual and motor systems (Brown et al., 2000; Feldeheim et al., 2000; Frisen and Barbacid, 1997; Frisen et al., 1998). In addition, Eph receptors and ephrins are expressed in developing and mature synapses in the nervous system, where they may have a role in regulating synaptic formation and plasticity (Dalva et al., 2000; Torres et al., 1998). Ephrins have been attributed the unique function of being repulsive cues for receptor-bearing axons by promoting the collapse of the actin cytoskeleton within the growth cone, thereby controlling axonal pathfinding (Gale and Yancopoulos, 1997). However, more recent studies including work from our laboratory indicate that these molecules could have both attractant as well as repulsive roles in cell migration and guidance (Davy et al., 1999; Davy and Robbins, 2000; Feldeheim et al., 2000; Holmberg et al., 2000; Huai and Drescher, 2001; Knoll et al., 2001). It should also be noted that the Eph receptors and their ligands are involved in other processes including angiogenesis and vascularization. During embryonic vascular network formation, the receptors and ligands provide cues for the molecular distinction between arteries and veins (Wang et al., 1998). Furthermore, they are also able to function as angiogenic factors in vivo (Pandey et al., 1995). Evidence is now gathering to suggest that dysregulation of the Eph-ephrin system could promote tumorigenesis (Dodelet and Pasquale, 2000).

![Fig. 1. Eph receptors and ephrin ligands participate in bi-directional signaling. Eph receptor tyrosine kinases are classified into two subfamilies, EphA and EphB, based on their sequence similarity and binding affinities for their respective ligands that are tethered to the cell surface by either a GPI-anchor (ephrinA) or by a single transmembrane domain (ephrinB). When engaged, the Eph receptors initiate a signaling cascade in the forward direction, and a signal is also transmitted in the ephrin expressing cells (reverse signal).](image-url)
Ephrins Kiss and Tell

From accumulating genetic and biochemical studies, it is quite evident that one of the unique features of the ephrin-Eph system is the ability to elicit a bi-directional signal, that is forward signaling by the Eph receptor via its intrinsic kinase activity and reverse signaling via the ephrins by non-classical means (Fig. 1) (Schmucker and Zipursky, 2001).

The first evidence that suggested that ephrins are able to signal independently from Eph receptors was observed with the B-class ephrins. Mice lacking the EphB2 RTK have defects in the guidance of the anterior commissure (Henkemeyer et al., 1996). However, axons are appropriately guided to form the anterior commissure in transgenic mice expressing a catalytically inactive Eph RTK, which supports the notion that the ephrinB ligands can actively guide these particular axons even in the absence of functional Eph receptor signaling (Henkemeyer et al., 1996). Others have also shown that reverse signaling through ephrinB is essential for proper pathfinding of dorsal retinal axons (Birgbauer et al., 2000). Consistent with the latter observation, it was recently established that the intracellular domain of ephrinB2 possesses an intrinsic reverse signaling function that is essential for proper vascular morphogenesis (Adams et al., 2001). With the use of an animal cap assay, bi-directional signaling between EphB-ephrinB was proven necessary to restrict cell intermingling (Mellitzer et al., 1999). Biochemical evidence also supports the ability of membrane-spanning ephrins to participate in bi-directional cell signaling (Fig. 2). The cytoplasmic tail is the most conserved region between all three B-class ephrins. In particular, the last 33 amino acids display a 95% identity between all three proteins. In this region there are five conserved tyrosine residues, a polyproline stretch, as well as a PDZ binding motif at the extreme C-terminus. Transmembrane ephrins were initially shown to be tyrosine phosphorylated in vitro by v-Src (Holland et al., 1996), and in response to stimulation of cultured cells by either clustered Eph B receptors or platelet-derived growth factor (PDGF) (Bruckner et al., 1997; Holland et al., 1996). Very recently, it has been shown that ectopic expression of ephrin B1 in 293T cells leads to JNK activation (Xu et al., 2003). This appears to occur in a manner, which is dependent upon the cytoplasmic tail of ephrin B1. Multiple in vivo tyrosine phosphorylation sites of ephrin B1 and Eph B2 have been mapped from developing murine retinal tissues (Kalo et al., 2001). This denotes a physiological significance to the ligands phosphorylation (Holland et al., 1996; Kalo et al., 2001). Grb4 has been demonstrated to be recruited in a tyrosine phosphorylated dependent manner to ephrin B1 via its SH2 domain (Cowan and Henkemeyer, 2001). Downstream cytoskeletal changes which ensue from this latter association are proposed to be mediated through adaptor protein functions of Grb4. The induced association of Dsh and ephrin B1 in Xenopus following clustered Eph B ectodomain stimulation has been demonstrated to result in activation of Rho kinase and RhoA GTPase (Tanaka et al., 2003). This further links ephrin B reverse signaling function with cytoskeletal changes. An assortment of PDZ-domain containing proteins have been found to interact with ephrin B1, which include PTP-BL (FAP-1) (Palmer et al., 2002), GRIP (Bruckner et al., 1999), and the novel PDZ-RGS3 (Lu et al., 2001), however their role in mediating a physiological response has not been determined as of yet. A hypothesized function of PDZ-domain containing protein is to be involved in assembling supramolecular signaling complexes (Harris and Lim, 2001). It is interesting to speculate that the association of PDZ-domain containing proteins with B-class ephrins may not only be required for their presently described role in their cellular signaling. But in addition, these proteins may be involved in a key feature of ephrin activation which is their assembly into clusters. The cytosolic domain of transmembrane ephrins has been implicated in modulating migration and cell attachment in an integrin-dependent fashion following Eph stimulation implicating the ligands in ‘inside-out’ signal transduction (Huynh-Do et al.,...
B-class ephrins can also be tyrosine phosphorylated in response to cellular stimulation with PDGF or through association with activated fibroblast growth factor (FGF) receptors (Bruckner et al., 1997; Chong et al., 2000). It has been demonstrated that in Xenopus ephrin B1 directly associates with activated FGF receptor and that this interaction requires the presence of a particular set of tyrosine residues of ephrin B1’s cytoplasmic tail (Chong et al., 2000). In contrast to Eph stimulation, ephrin B tyrosine phosphorylation following PDGF addition does not seem to involve Src family kinases (Bruckner et al., 1997). Thus, growth factor and Eph receptor stimulation of ephrins appear to function through distinct mechanisms. A potential dynamic factor, which may be important in the specificity of B-class ephrin signaling, is to undergo a conformational change. The solution structure of the last 33 amino acids of ephrin B2’s cytoplasmic tail indicated that it possesses two distinctive structural features (Song et al., 2002). Following Eph receptor stimulation, this highly conserved region found in all B-class ephrins may succumb a conformational change induced by tyrosine phosphorylation (Song, 2003), consequently serving to recruit in various proteins to modulate the activation of the ligand. With respect to their
downregulation, thus far only PTP-BL, a PDZ-domain containing protein with a protein tyrosine phosphatase domain, has been shown to dephosphorylate the B-class ephrins (Palmer et al., 2002).

Although initially thought to be unable to transduce signals due to their mode of membrane attachment (GPI-moiety), it has since been established that not unlike their ephrinB kin, A-class ephrins are themselves able to communicate signals inside the cell when stimulated by Eph receptors. Genetic studies in C. elegans have demonstrated that their ephrin homologues, all of which contain a GPI modification, have functions independent of their cognate Eph receptor (VAB-1). In this model genetic organism, it has been observed that mutation of ephrins synergizes with VAB-1 kinase domain mutations, and that VAB-1 kinase domain mutants exhibit a weaker mutant phenotype than VAB-1 ligand binding domain mutants, suggesting that the GPI-tethered ligands, like the transmembrane ligands can be involved in reverse signaling in vivo (Chin-Sang et al., 1999; Wang et al., 1999). Based on our previous work demonstrating that the GPI-anchored protein CD59 could transmit an intracellular signal (Murray and Robbins, 1998), we proposed that the GPI-anchored ephrins were also competent of communicating an intracellular signaling response. We have now shown that ephrinA5 is able to induce a signaling response within the ephrinA-expressing cell when bound to its cognate Eph receptor (Davy et al., 1999; Davy and Robbins, 2000) (Fig. 2). Fyn, a member of the Src family of non-receptor tyrosine kinases, is a downstream signaling protein targeted in activated ephrin A5 cells. Furthermore, we and others have demonstrated a physiological consequence to the ephrinA-induced signaling event, namely a change in cell adhesion and morphology that is modulated by integrins (Davy et al., 1999; Davy and Robbins, 2000; Huai and Drescher, 2001). A potential mode of downregulation for A-class ephrin signaling has been proposed which involves the cleavage of these molecules by the Kuzbanian metalloprotease (Hattori et al., 2000). Although this likely represents a means to downregulate ephrinA-mediated signaling, it is possible that following the cleavage that the remaining inositol phosphoglycan (IPG) moiety could act as an active second messenger and modulate intracellular signaling events. Evidence for this unique signaling paradigm arises from studies on insulin-mediated signaling where cleavage of GPI-anchored proteins, presumably by a phospholipase C-like activity, results in the production of IPG which subsequently modulates certain aspects of insulin signaling (Lazar et al., 1994).

Ephrins are localized within membrane microdomains

In addition to their common characteristics of being involved in ‘inside-out’ mediated integrin signaling, and that Src family kinases have been implicated in their signal transduction pathways, both classes of ephrins also share the property of initiating signals from specific signaling ‘hot spots’ found on the plasma membrane. As is the case for many GPI-anchored proteins (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998) ephrinA5 is localized within discrete membrane microdomains known as lipid rafts (Davy et al., 1999). Furthermore, the signaling that is initiated by ephrinA-expressing cells is compartmentalized within these domains (Davy et al., 1999). Independently, it has been shown that the transmembrane ephrins are also localized within lipid rafts (Bain et al., 1995; Bruckner et al., 1999). Lipid rafts are liquid-ordered membrane microdomains which represent an assembly of glycosphingolipids and cholesterol (Simons and Ikonen, 1997). These specialized membrane microdomains have a role in a wide variety of processes, including: 1) transcytosis (Simionescu, 1983), 2.) potocytosis (Anderson et al., 1992), 3.) alternate route of endocytosis (Parton et al., 1994), 4.) internalization of toxins, bacteria and viruses (Fivaz et al., 1998; Shin and Abraham, 2001; Shin et al., 2000), 5.)
cholesterol transport (Smart et al., 1996), 6.) calcium homeostasis (Isshiki and Anderson, 1999), 7.) protein sorting (Simons and Ikonen, 1997) and 8.) signal transduction (Anderson, 1998; Simons and Toomre, 2000). It has been hypothesized that lipid rafts can serve as sites of signal integration largely based on the observation that many molecules known to be involved in intracellular signaling are enriched within them (for review see (Anderson, 1998; Simons and Toomre, 2000; Zajchowski and Robbins, 2002)). For instance, we and others have shown that Src-family kinases, important mediators for a variety of signal transduction pathways, are localized within these membrane domains (Robbins et al., 1995; Shenoy-Scaria et al., 1994). Various receptor-mediated signaling systems, including various growth factor receptors and immune receptors, are thought to utilize such membrane compartmentalization to elicit a functional signaling response (Anderson, 1998; Zajchowski and Robbins, 2002). Regulated signal transduction in these lipid rafts is an attractive hypothesis for achieving spatial and temporal specificity in signaling for which the ephrins may be excellent examples.

We have proposed that the mode of membrane attachment (i.e. GPI vs. transmembrane) defines signaling specificity for each class of ephrin. Interestingly, Stanners and colleagues have shown that the ability of the human carcinoembryonic antigen (CEA) family of adhesion receptors to block or promote myogenic differentiation is imparted by the mode of membrane attachment (Screaton et al., 2000). The GPI-anchored CEAs (CEA, CEACAM6) block differentiation whereas, the transmembrane isoforms (CEACAM1) promote differentiation. Replacement of the transmembrane domain with a GPI moiety is sufficient to convert CEACAM1 into a differentiation blocking protein (Screaton et al., 2000). Since we have shown that the GPI-anchored ephrins reside within lipid rafts our initial hypothesis was that the GPI moiety selectively targets this class of ephrins to these domains, while the transmembrane ephrins are excluded from these specialized membrane domains. This differential membrane localization would then facilitate the interactions with distinct signaling effectors, providing the basis for the induction of unique physiological responses. This view was erroneously simplistic, since other researchers have demonstrated that ephrinBs are also localized within lipid rafts (Bain et al., 1995; Bruckner et al., 1999; Palmer et al., 2002), implying that selective membrane localization could not explain the intrinsic differences in signaling capabilities. However, based on the observation that there are different types of lipid rafts on each individual cell (Madore et al., 1999) we have modified our hypothesis to suggest that ephrinA and ephrinB reside in functionally distinct lipid rafts, each of which couples them to discrete downstream effector pathways.

There is now compelling evidence to suggest that there are distinct subpopulations of lipid rafts on the same cell. This data includes the localization of various GPI-anchored proteins into distinct membrane microdomains (Madore et al., 1999). In addition, at least two distinct populations of lipid rafts appear to be present on the apical plasma membrane of epithelial cells: one is localized to the microvilli containing the raft-associated transmembrane protein prominin, and a second population containing the GPI-anchored protein PLAP, which did not co-localize with prominin by immunofluorescence (Roper et al., 2000). This coupled with the observation that caveolae, a specific subtype of lipid raft that is morphologically distinguishable as a plasma membrane invagination, are present on the basolateral surface of epithelial cells (Scheiffele et al., 1998; Vogel et al., 1998) suggests that at least three distinct types of lipid rafts may be present in these cells. Electron microscopy studies of signaling molecules downstream of FcεRI in resting and activated mast cells suggest that distinct membrane domains exist in mast cells as well (Wilson et al., 2001). It is interesting to note that distinct populations of lipid rafts are required for the acquisition of polarity during T cell chemotaxis, in which the protruding leading edge and rear uropod of lymphocytes are enriched in specific signaling
molecules but lack others (Gomez-Mouton et al., 2001). In polarized migrating T cells, raft molecules GM1 and CD44 colocalize at the uropod, whereas rafts enriched in GM3, talin, the chemokine receptor CXCR4 and uPAR were detected at the leading edge (Gomez-Mouton et al., 2001). Raft association of these membrane proteins was key for their asymmetric distribution, and this process also required an intact cytoskeleton (Gomez-Mouton et al., 2001). Taken together, these observations beg the question as to whether the two classes of ephrins are localized in distinct lipid rafts. If so, what is the molecular composition of these lipid rafts and do the different ephrin containing lipid rafts have discrete functions? Currently it is very difficult to define whether distinct lipid rafts have unique functions since our ability to disrupt one versus the other is inadequate thus far; we are restricted to fairly barbaric means of depleting membrane cholesterol (Keller and Simons, 1998) or adding various polyunsaturated fatty acids (Stulnig et al., 1998; Stulnig et al., 2001; Webb et al., 2000) in order to disrupt or alter lipid raft function. This is a rapidly emerging field and based on the identification of new lipid raft markers and various new microscopy techniques this issue should be resolved shortly. However, it is important to consider that many of these studies rely solely on in vitro systems and the final proof will be in defining a role for compartmentalized signaling in vivo.

Conclusion

It is now clear from both genetic and biochemical studies that the ephrins are more than just ligands for the Eph receptors and possess the intrinsic capacity to signal. Moreover, there are indications that the ephrin-mediated signaling events are initiated within specialized plasma membrane microdomains, known collectively as lipid rafts. We propose that in contrast to Tolkien’s classic tale of the Dark Lord’s ring, that there is more than one type of lipid raft found in a cell to ‘rule’ over signaling. Furthermore, with respect to the specificity of ephrin signaling, we hypothesize that the association of the different classes of these ligands within distinct populations of lipid rafts is a dynamic factor involved in defining and transmitting their unique signals inside the cell to elicit a specific physiological response.

Acknowledgements

Work from the Robbins laboratory is supported by grants from the Canadian Institutes of Health Research and the Cancer Research Society. LRG is supported by a graduate fellowship from the Alberta Heritage for Medical Research Foundation (AHFMR) and SMR is an AHFMR Senior Scholar and currently holds a Canada Research Chair in Cancer Biology.

References


