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ErbB receptors and tetraspanins: casting the net wider.

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Two decades ago it was first proposed that four transmembrane domain proteins of the tetraspanin superfamily function as membrane adaptors (or facilitators) for protein interactions (1; 2). Ever since, researchers have been trying to find a common ground which would explain how these small proteins affect so many seemingly unrelated processes such as cell-cell fusion, antigen presentation and receptor mediated signal transduction. Up until recently, the widely held view was that tetraspanins connect various protein assemblies on the membrane thus forming an interlinked network of functional hubs often referred to as tetraspanin-enriched microdomains or tetraspanin webs (3). However, the idea of tetraspanin-based networks has been recently challenged in the report which demonstrated, using super resolution microscopy, that various tetraspanins are localized in separate, mostly non-overlapping nanoclusters (4). Whilst this observation may pave the way for the reassessment of a more general model of tetraspanin microdomain organisation, the conceptual view of tetraspanins as regulators of compartmentalization and clustering of the associated receptors remains the main investigative theme.

In this short review we will specifically focus on the links between tetraspanin proteins and ErbB receptors (Fig.1). For a more general overview of how various types of membrane microdomains regulate activities of receptor tyrosine kinases readers are referred to an excellent recent review article by Delos Santos and colleagues (5).

ErbB receptors

ErbB receptors are four monomeric receptor tyrosine kinases (i.e. EGFR/ErbB1, ErbB2/Her2, ErbB3 and ErbB4), which are activated by soluble and membrane-bound EGF ligands. Ligand binding induces homo- and heterodimerization of the receptors leading to phosphorylation of their cytoplasmic regions at multiple tyrosine residues, assembly of a variety signalling complexes and activation of signalling networks that control cell proliferation, migration, survival and differentiation (6). Signalling via ErbB proteins is controlled at various levels including such steps as the receptor clustering, post-endocytic trafficking and sorting, and lysosomal degradation. Post-biosynthetic trafficking and proteolytic-based maturation of EGF ligands diversify the ErbB-targeting regulatory network even further. Among various modulators of the ErbB signalling network tetraspanins occupy a unique spot as effectors that target both the receptors and ligands (Fig.1). Below we focused on tetraspanins whose involvement in ErbB-centred signalling networks was clearly established.

Tetraspanins and ErbB receptors.

Recruitment of ErbB proteins to complexes with tetraspanins is known to alter basal and ligand-induced dimerization of the receptors, their endocytic

trafficking and signal transduction, thus suggesting a regulatory role for tetraspanins.

CD9 and ErbB receptors. CD9 can be co-immunoprecipitated with EGFR and β1 integrins from various cancer cell lines (7-9) . A steady-state level of EGFR was negatively regulated by CD9, and antibody-induced ligation of CD9 facilitated ligand-induced endocytosis of the receptor (7; 10). The surface clearance of EGFR was linked to CD9-dependent regulation of the expression of dynamin-2, a critical component of various endocytic pathways (10). Ligand-induced phosphorylation of EGFR and its partner adaptor protein Shc was decreased in CD9-expressing cells when compared to control, and CD9 was also shown to attenuate EGFR-induced activation of PI3-kinase and MAPK/Erk pathways (7; 11).

CD82 and ErbB receptors. Data generated in various laboratories strongly support the notion that CD82 acts as a negative regulator of EGFR signalling. We have initially reported that CD82 can be co-immunoprecipitated with EGFR, ErbB3 and ErbB2 (12; 13). An increase in the expression of CD82 in mammary epithelial cells did not affect ligand binding to EGFR but attenuated ligand-induced homodimerization of the receptor and EGFR-ErbB2 heterodimerization. This effect on dimerization was specific for EGFR-containing dimers as ErbB2-ErbB3 dimers were not affected (13). CD82 also accelerated ligand-induced internalization of EGFR and affected the kinetics of EGFR-triggered phosphorylation of downstream cellular targets. Danglot and colleagues used a cervical carcinoma cell line to examine effects of CD82 knockdown on internalization and activation of EGFR (14). In this study CD82 depletion restrained receptor diffusion and allowed more efficient recruitment of AP-2 adaptor to the EGF-activated receptor thus facilitating clathrin-mediated endocytosis of EGFR. They also found that the basal surface level of EGFR was decreased in CD82-depleted cells. Thus, both of these studies demonstrated that the expression level of CD82 may be an important factor that controls cell-type specific endocytic trafficking and signaling via EGFR.

Variations in the composition of CD82-centred microdomains may contribute further to cell type specific modifications of the endocytic machinery involved in the internalization of activated EGFR. In this regard, we have demonstrated that there is a correlation between CD82 expression level and levels of a-series gangliosides (15), glycosphingolipids that are known to control various aspects of signalling via ErbB receptors (16-19). Furthermore, we have shown that the stability of CD82-EGFR interactions is dependent on gangliosides. Specifically, pharmacological depletion of gangliosides or sialidase-dependent changes in ganglioside profile in CD82-expressing cells resulted in weakening of CD82-EGFR interactions and in reduction of EGFR phosphorylation in response to EGF (15). Interestingly, interactions of CD9 with EGFR were not affected in these experiments indicating dissimilarities between different tetraspanin-EGFR complexes. Wang and colleagues demonstrated that the inhibition of EGFR signaling through tetraspanin CD82 and ganglioside GM3 required PKC-α translocation to the plasma membrane and phosphorylation of $EGFR^{Thr654}$ (20). In the study GM3, which belongs to a-series gangliosides, facilitated the association of EGFR with caveolin-1, CD82 and PKC-α. These authors found that CD82 was critical for the recruitment of PKC-α into the complex and activation of the enzyme. More recently, we demonstrated that

PKC-α recruitment to tetraspanin-enriched microdomains, and its activation by HSPG affected interactions of EGFR with c-Cbl, a principal E3 ubiquitin ligase responsible for the ligand-induced ubiquitylation of the receptor (21). Importantly, ubiquitylation of EGFR by EGF ligands containing a heparin binding domain was attenuated in the presence of CD82. We also found that postendocytic trafficking of EGFR was diversified in the presence of CD82. The involvement of CD82 in the regulation of EGFR was also suggested in a recent study of non-small cell lung cancers (NSCLC). Analysis of NSCLC cell lines and samples from the patients indicated that activating mutations in EGFR are directly linked to downregulation of CD82. Whilst not investigated at the molecular level, the underlying mechanisms were proposed to involve EGFR-dependent release of CD82 via exosomes (22).

CD151 and ErbB receptors. Tetraspanin CD151 forms stoichiometric complexes with laminin-binding integrins (i.e. α3β1, α6β1/β4, α7β1), and most CD151-dependent cellular phenomena can be explained by CD151-integrin interactions. In earlier studies depletion of CD151 from breast cancer cells attenuated EGF-induced phosphorylation of FAK, Erk1/2 and β4 integrin subunit (23). Furthermore, ErbB2-driven tumourigenesis and metastasis was suppressed in animals lacking CD151 (24). These authors also reported that the inhibitory effect of ErbB2-targeting therapeutics (Herceptin and lapatinib) was accentuated in CD151-depleted cells plated on laminins (23). By contrast, we found that the growth suppressive effect of Herceptin was negligible when CD151-depleted cells were placed in laminin-rich extracellular matrix (25). In this experimental setting CD151 increased homodimerisation of ErbB2 via mechanisms involving integrin-dependent activation of RhoA GTPase (25). It is likely that CD151 affects ErbB signalling indirectly, as in neither of these studies was a physical association between CD151 and ErbB proteins observed.

CD81 and ErbB receptors. Functional links between CD81 and EGFR were studied in the context of hepatitis C virus (HCV) infection. HCV binding and crosslinking with anti-CD81 mAb induced phosphorylation of EGFR and co-internalisation of the receptor and tetraspanin into EEA-1 – positive endosomes (26). These results suggested that EGFR activation is required for HCV entry at a step occurring soon after CD81 and/or CLDN1 binding. Furthermore, although the authors were unable to detect CD81-EGFR association in co-immunoprecipitation experiments, they proposed that HCV-induced CD81 crosslinking induced ligand-independent dimerization and activation of the receptor. More recently, it was reported that CD81 also plays a role in activation of Raf-1, a well-established down-stream target in EGFR-dependent HCV entry. Basal phosphorylation of Raf-1 on Ser259 and Ser338 could be modulated by clustering with anti-CD81 mAb (27). Given that phosphorylation of these residues has an opposing effect on Raf-1 activation, CD81 is likely to function as a key switch that controls the dynamics of the kinase activity during HCV infection.

Tetraspanins and ErbB ligands.

Interaction of ErbB ligands with tetraspanins. Transmembrane (tm) forms of HB-EGF and TGFα were identified as partners for CD9 (28; 29). Whilst it is unlikely that the interaction is direct (see also below), it has been shown that

CD9 regulates HB-EGF – and TGFα–induced juxtacrine activation of EGFR (29; 30). Furthermore, CD9-dependent potentiation of tm-TGFα juxtacrine function may be due to changed surface distribution of the protein (31).
Proteolytic shedding. All EGF-like ligands for ErbB receptors are synthesized as transmembrane precursor proteins and subsequently cleaved/shed by ADAM17 and ADAM10, transmembrane metalloproteases of the ADAM superfamily (32). ADAM17 was shown to directly interact with the LEL CD9 (33). Although the role of CD9 in modulation of the enzyme activity towards EGF ligands has not been investigated other studies demonstrated that cleavage of three of its other substrates was negatively regulated by the tetraspanin. ADAM10 was reported to be the main sheddase for betacellulin and EGF (34). In the initial report describing the ADAM10-tetraspanin interactions, antibodies to CD9, CD81 and CD82 were shown to increase shedding of EGF (35). Subsequent studies demonstrated that ADAM10 is recruited to tetraspanin enriched microdomains via TspanC8 tetraspanins which regulate various aspects of ADAM10 intracellular trafficking and control activity of the enzyme towards its multiple substrates (36-38).

Future directions.
ErbB proteins and their ligands are released by cells via exosomes, extracellular vesicles (30-100nM) secreted by all cell types (39). Importantly, exosome-associated ErbB ligands are more potent in stimulating proliferative and migratory cellular responses (40). Several tetraspanins are known to control production and composition of exosomes (41), and, therefore, they are expected to play an important role in exosome-dependent activation of ErbB receptors.

The involvement of tetraspanins in proteolytic shedding of surface proteins of the ErbB signalling network will undoubtedly gain further momentum in the future. Tetraspanins are associated with ADAM proteases and other metalloproteinases (e.g. MT1-MMP and MMP7) (42). Not only do these proteases target various ErbB ligands, but they are also responsible for the proteolytic cleavage of ErbB receptors themselves (43; 44). For example, shedding of ErbB2 by ADAM10 is proposed to be one of the mechanisms of resistance to Herceptin/Trastuzumab-based therapies in patients with breast cancer (43). Thus, TspanC8-dependent regulation of the activity of ADAM10 towards ErbB2 can be predicted. There is also an intriguing possibility that TspanC8 activity towards ADAM10 is further regulated by other tetraspanins. It has been reported that CD63 binds TIMP1, a well-established inhibitor of ADAM proteases (45). This lends a possibility for a spatial, tetraspanin-dependent control over activation of ADAM proteins which may represent an important step in one of the recently described "bypass" signaling pathways involving ErbB-centred signalling network (46).

Tetraspanins and ErbB proteins are engaged in N-linked glycans – mediated interactions with gangliosides which control various aspects of ErbB-dependent signalling. Given that tetraspanins are able to modify a glycosylation pattern of the associated receptors (47) and cellular levels of gangliosides (13), it is likely that saccharide-mediated interactions could be one of the important factors in fine tuning of signalling via ErbB proteins. Thus, detailed characterisation of tetraspanin-dependent changes in ErbB

glycotopes will lead to better and more complete understanding of how tetraspanins regulate ErbB-dependent signalling networks.

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Schematic representation of ErbB-centered pathways affected by tetraspanins