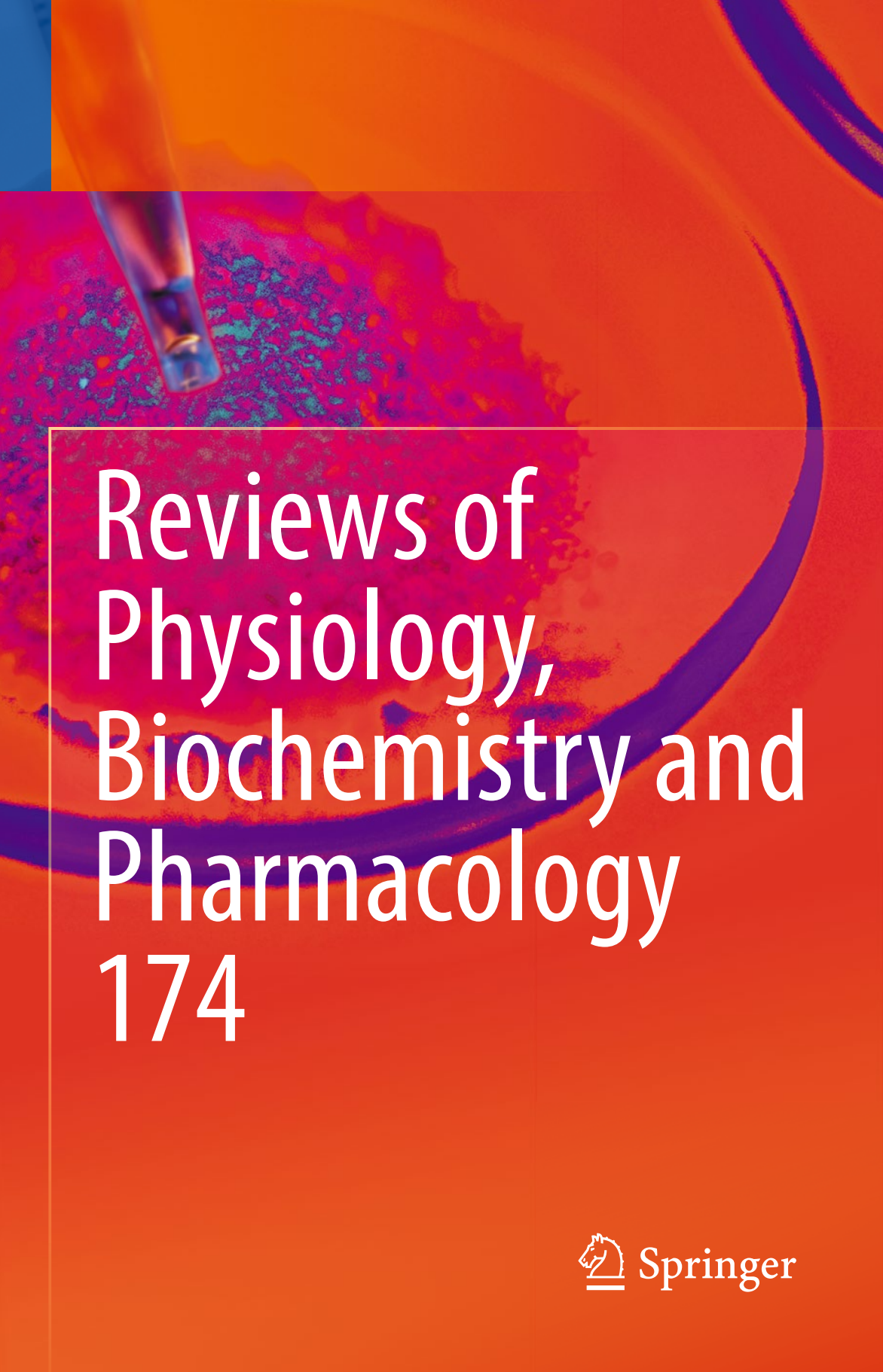
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Ezrin Orchestrates Signal Transduction in Airway Cells

Lei-Miao Yin, Ting-Ting Duan, Luis Ulloa, and Yong-Qing Yang

Abstract Ezrin is a critical structural protein that organizes receptor complexes and orchestrates their signal transduction. In this study, we review the ezrin-meditated regulation of critical receptor complexes, including the epidermal growth factor receptor (EGFR), CD44, vascular cell adhesion molecule (VCAM), and the deleted in colorectal cancer (DCC) receptor. We also analyze the ezrin-meditated regulation of critical pathways associated with asthma, such as the RhoA, Rho-associated protein kinase (ROCK), and protein kinase A (cAMP/PKA) pathways. Mounting evidence suggests that ezrin plays a role in controlling airway cell function and potentially contributes to respiratory diseases. Ezrin can participate in asthma pathogenesis by affecting bronchial epithelium repair, T lymphocyte regulation, and the contraction of the airway smooth muscle cells. These studies provide new insights for the design of novel therapeutic strategies for asthma treatment.

Keywords Actin-binding proteins • Airway cells • Asthma • Ezrin

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Contents

1 Introduction

Asthma is a clinical challenge in modern medicine that affects over 300 million people and causes over 250,000 deaths annually worldwide (D'Amato et al. 2016; Lambrecht and Hammad 2015). Structural airway cells, such as smooth muscle and epithelial cells, are critical factors that contribute to asthma. Multiple studies suggest that alterations in the actin cytoskeleton cause a pathological contraction of the structural airway cells, which contributes to asthma (Fletcher and Mullins 2010; Noble et al. 2014). In clinical studies, biopsied tissue from asthmatic patients showed reduced β-actin mRNA levels (Glare et al. 2002). Altogether, these results encouraged investigators to study the actin-binding proteins in the airway cells and their clinical implications in asthma (Tang 2015).

This review analyzes recent studies on ezrin and its potential implications in asthma. Ezrin is a principal member of the ERM (ezrin–radixin–moesin) protein family, which includes actin-binding proteins of the band 4.1 superfamily because their N-termini are similar to those of the erythrocyte cytoskeletal protein band 4.1 (Sagara et al. 1995; Vaheri et al. 1997; Gould et al. 1989; Ng et al. 2001). The ERM proteins are known as structural organizers that link membrane proteins to the underlying actin cytoskeleton. In addition to their structural role, the ERMs also regulate the interaction between receptor complexes and intracellular proteins, thereby modulating signal transduction pathways, such as the RhoA, Rho-associated protein kinase (ROCK), and cyclic AMP/protein kinase A (cAMP/PKA) pathways (Iontcheva et al. 2004; Ponta et al. 2003; Celik et al. 2015). The ERMs are expressed in a developmental and tissue-specific pattern. Ezrin is mainly expressed in lymphocytes and epithelial cells, moesin is expressed in endothelial cells, and radixin is expressed in hepatocytes. Ezrin has recently attracted the

attention of multiple investigators due to its role in key biological processes such as the immunological synapsis in T lymphocytes and the epidermal growth factor (EGF)-induced stimulation of human carcinoma tumor differentiation and metastasis (Bretscher et al. 1997; Yoshida et al. 2016). The interaction between ezrin and various receptor complexes and intracellular targets is mainly regulated by phosphorylation (Neisch and Fehon 2011). In this study, we review recent results of ezrin regulation and its physiological and clinical implications.

2 Molecular Features of Ezrin

2.1 Discovery of Ezrin

Ezrin was discovered in multiple cellular processes and was thought to be different proteins because of its different electrophoretic mobility on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Gould et al. 1989; Chambers and Bretscher 2005). Ezrin was first identified in 1981 as a polypeptide with an apparent molecular weight of 81-kDa on SDS-PAGE that was quickly phosphorylated after the stimulation of *human* A431 carcinoma cells with EGF (Hunter and Cooper 1981). In 1983, ezrin was purified as a polypeptide with an apparent molecular weight of 80-kDa on SDS-PAGE from the microvillus cytoskeleton in *chicken* intestinal epithelial cells (Bretscher 1983). In 1986, both polypeptides were compared and identified as ezrin (true molecular weight of 69-kDa), which was confirmed by immunoblotting, immunoprecipitation, two-dimensional gel electrophoresis, and protein sequencing (Gould et al. 1986). Ezrin was also purified as an 82-kDa tumor antigen from the cytosol of a methylcholanthrene-induced sarcoma (Ullrich et al. 1986; Fazioli et al. 1993). In 1988, ezrin was isolated from the microvillar membranes of *human* choriocarcinoma cells as a 75-kDa protein named cytovillin (Pakkanen et al. 1987; Turunen et al. 1989). Ezrin was also purified as a 78-kDa cyclic AMP-dependent kinase anchoring protein (originally named AKAP78) enriched in *murine* gastric parietal cells (Dransfield et al. 1997). These studies showed that ezrin is involved in multiple cellular processes ranging from the EGF stimulation of *human* carcinoma cells to tumor antigens. These results also indicated that the original confusion was due to the different electrophoretic mobility of ezrin on SDS-PAGE (Gould et al. 1989). These differences were mostly due to its phosphorylation, indicating that ezrin is regulated by phosphorylation, which induces structural changes that affect its electrophoretic mobility.

2.2 *Ezrin Phosphorylation*

Ezrin contains an N-terminal FERM (four-point one, ezrin, radixin, moesin) domain (~300 residues), a central linker region (~200 residues), and a C-terminal ERM-associated domain (C-ERMAD, ~80 residues; Fig. 1) (Jayasundar et al. 2012). The N-terminal FERM domain consists of the following three subdomains: F1–F3. These subdomains have structural (but not sequence) homology to known folded proteins. F1 is similar to ubiquitin, F2 is similar to acyl-CoA-binding protein, and F3 is similar to the PTB (phosphotyrosine binding)-domain. All ERMs have a central helical linker region composed of the predicted α-helical domain. Ezrin and radixin, but not moesin, have a proline-rich linker domain (~470–497 residues) preceding the C-ERMAD. Ezrin is normally a dormant inactive protein due to the intramolecular interaction between the N- and C-terminal domains. Ezrin is activated by phosphorylation, which dissociates the intramolecular interaction between the N- and C-terminal domains and allows the N-terminal domain to interact with membrane receptor complexes and the C-terminal domain to interact with F-actin (Bretscher et al. 1997). Thus, the interaction between ezrin and other proteins is regulated by its phosphorylation in multiple domains by various kinases (McRobert et al. 2003; Fehon et al. 2010). A detailed list of the ezrin phosphorylation sites, kinases, and biological activity is provided in Table 1.

The most common ezrin phosphorylation site is threonine-567 in the C-terminal domain (Zhu et al. 2007). Ezrin threonine-567 is phosphorylated by calcineurin homologous protein-1 (CHP1), and the inhibition of CHP1 abrogates the interaction between ezrin and the Na^+/H^+ exchanger 3 (Di Sole et al. 2009). The phosphorylation

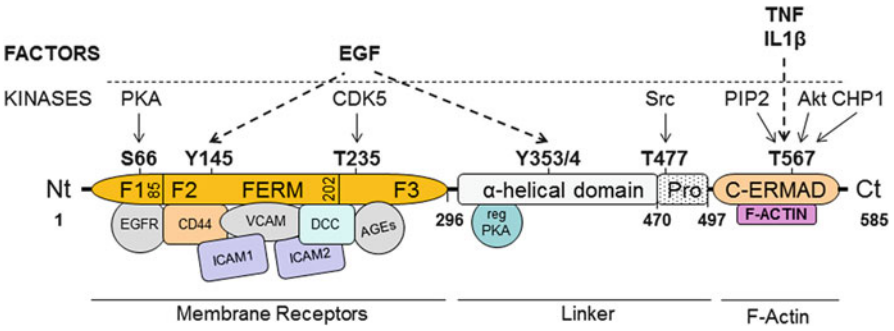

Fig. 1 Structural characteristics of ezrin. Ezrin contains an N-terminal FERM domain (~300 residues), a central linker region (~200 residues), and a C-terminal ERM-associated (C-ERMAD) domain. The N-terminal domain binds to membrane receptor complexes. The linker is an α-helix with a proline-rich domain. The C-terminal C-ERMAD domain binds to F-actin. Ezrin is regulated by phosphorylation at serine-66, tyrosine-145, threonine-235, tyrosine-353, threonine-477, and threonine-567. These phosphorylations are regulated by multiple extracellular factors (EGF, TNF, and IL1β) and intracellular kinases (PKA, CDK5, Src, and Akt). *FERM* four-point one, ezrin, radixin, moesin, *EGF* epidermal growth factor, *TNF* tumor necrosis factor, *IL1β* interleukin-1β, *PKA* protein kinase A, *CDK5* cyclin-dependent kinase 5, *SRC* proto-oncogene tyrosine-protein kinase Src, PIP_2 phosphatidylinositol 4,5-bisphosphate, *CHP1* calcineurin homologous protein-1

Table 1 Phosphorylation sites for the ezrin activation

Phosphorylation site	Kinases/ phosphorylation factors	Cell line	Biological activity	References
Threonine-567	Phosphatidylinositol 4,5-bisphosphate (PIP_2)	LLC-PK1 epithelial cell line	Epithelial cell morphogenesis	Fievet et al. (2004)
Threonine-567	Calyculin A	LLC-PK1 epithelial cell line	Cytoskeleton arrangement and development of multicellular epithelial structures	Gautreau et al. (2000)
Threonine-567	Calcineurin homologous protein-1	Opossum kidney cells	Na^+ transport	Di Sole et al. (2009)
Threonine-567	TNF-α and IL-1β	Human synoviocytes	Migration and invasion of fibroblast-like synoviocytes	Xiao et al. (2014)
Threonine-567	Akt	BeWo trophoblastic cells from human choriocarcinoma	Microvilli-mediated mechanoresponsive cellular functions, such as epithelial absorption, signal perception, and mechanotransduction	Miura et al. (2015)
Tyrosine-353	Akt (but not ERK1/2, ROCK1) pathway	Tongue squamous cell carcinomas (TSCC) cell	Metastasis of TSCC cells	Wang et al. (2014)
Threonine-567/ Tyrosine-353	N/A	Pancreas tissue	Associated with positive lymph node metastasis, less differentiation, pAkt overexpression, and shorter survival times	Cui et al. (2010)
Threonine-567/ Tyrosine-353	N/A	Tissue of intraductal papillary mucinous neoplasms (IPMNs) and pancreatic intraepithelial neoplasia (PanINs)	Associated with tumor invasion and related to early development of PanINs	Oda et al. (2013)
Tyrosine-145/ Tyrosine-353	EGFR (epidermal growth factor receptor)	Human epidermoid carcinoma A431 cell	Unclear	Krieg and Hunter (1992)

(continued)

Table 1 (continued)

Phosphorylation site	Kinases/ phosphorylation factors	Cell line	Biological activity	References
Threonine-235	CDK5 (cyclin-dependent kinase 5)	Human osteosarcoma cell line SAOS-2	pRb activity and cytoskeletal regulation	Yang and Hinds (2003)
Threonine-235	CDK5	Human osteosarcoma cell line SAOS-2, senescent human diploid fibroblasts	Prevent senescence-associated flat cell formation	Yang and Hinds (2006)
Serine-66	PKA	Gastric parietal cells	Remodeling of the apical membrane cytoskeleton associated with acid secretion	Zhou et al. (2003)
Threonine-477	Src	Human embryonic kidney 293 cells	Unclear	Heiska and Carpen (2005)

of threonine-567 via phosphatidylinositol 4,5-bisphosphate (PIP_2) is necessary for ezrin activation during kidney epithelial LLC-PK1 cell morphogenesis (Fievet et al. 2004). Ezrin threonine-567 is also phosphorylated by Akt, which induces microvilli in *human* BeWo trophoblastic cells (Miura et al. 2015). The phosphorylation of threonine-567 enhances the binding of ezrin to the actin cytoskeleton and is crucial for establishing epithelial polarity in epithelial LLC-PK1 cells (Gautreau et al. 2000). The phosphorylation of ezrin threonine-567 contributes to the migration and invasion of fibroblast-like synoviocytes in rheumatoid arthritis (Xiao et al. 2014). The ezrin C-terminal domain is also regulated by the phosphorylation of threonine-477. The inhibition of *Src* by the PP2 chemical inhibitor prevented the phosphorylation of ezrin threonine-477 that is induced by pervanadate in *human* embryonic kidney 293 cells (Heiska and Carpen 2005). The phosphorylation of threonine-477 by *Src* enhances the binding of ezrin to the Kelch-repeat and BTB/POZ domain containing 2 (KBTBD2) complex, which suggests that ezrin regulates cellular morphology and adhesion (Heiska and Carpen 2005).

The phosphorylation of the N-terminal domain regulates the binding of ezrin to a variety of proteins and membrane complexes. PKA phosphorylates ezrin serine-66 during the remodeling of the apical membrane during the acid secretion of gastric parietal cells (Zhou et al. 2003). CDK5 (cyclin-dependent kinase 5) induces the phosphorylation of ezrin threonine-235, which regulates the cellular shape and cytoskeletal activity in *human* osteosarcoma SAOS-2 cells (Yang and Hinds 2003). The phosphorylation of threonine-235 by CDK5 causes ezrin to dissociate from the Rho GDP-inhibitor (Rho-GDI) and prevents senescence-associated flat cell formation in SAOS-2 cells and *human* diploid fibroblasts (Yang and Hinds 2006).

Ezrin is also regulated by tyrosine phosphorylation in both the N-terminal domain (Y145) and the helical linker (Y353/4) (Fehon et al. 2010). No tyrosine phosphorylation has been reported in the C-terminal domain, suggesting that tyrosine phosphorylation does not regulate the binding of the ezrin C-terminal domain to F-actin (Fehon et al. 2010). As mentioned above, ezrin was originally identified as a protein that was quickly phosphorylated at tyrosine-353 after the stimulation of *human* epidermoid carcinoma A431 cells with EGF (Hunter and Cooper 1981; Krieg and Hunter 1992). Ezrin is phosphorylated at both tyrosine-145 and 353 during carcinoma differentiation and invasion (Saygideger-Kont et al. 2016; Bretscher 1989). The phosphorylation of ezrin tyrosine-353 is related to tumor differentiation associated with positive lymph node metastasis and shorter survival times in *human* invasive pancreatic carcinomas (Wang et al. 2014; Fehon et al. 2010; Cui et al. 2010). The phosphorylation of ezrin tyrosine-353 by Akt (but not ERK1/2 or ROCK1) has been associated with the metastasis of tongue squamous cell carcinomas (Wang et al. 2014). This phosphorylation induced invasive ductal carcinoma in *human* pancreatic intraepithelial neoplasia (Oda et al. 2013). These results indicate that ezrin is regulated by multiple kinases (PKA, Akt, and *Src*) during critical cellular processes including carcinoma tumor differentiation, survival, and metastasis.

3 Ezrin Counterparts: From Receptors to Scaffold Proteins

Ezrin is considered a key regulator of airway cells that modulates the membrane receptor complexes and their signal transduction pathways (Neisch and Fehon 2011; Miura et al. 2015; Perez-Cornejo et al. 2012; Fievet et al. 2007). Ezrin is expressed in airway cells, including both epithelial and smooth muscle cells, and interacts with receptor complexes via its N-terminal domain and the F-actin cytoskeleton via its C-terminal domain (Miura et al. 2015). A schematic of the interaction between ezrin and the membrane receptor complexes is shown in Fig. 2. These interactions are critical for modulating receptor localization, complex organization, and signal transduction pathways. Ezrin regulates critical protein including epidermal growth factor receptor (EGFR), CD44, vascular cell adhesion molecule (VCAM), and deleted in colorectal cancer (DCC) receptor.

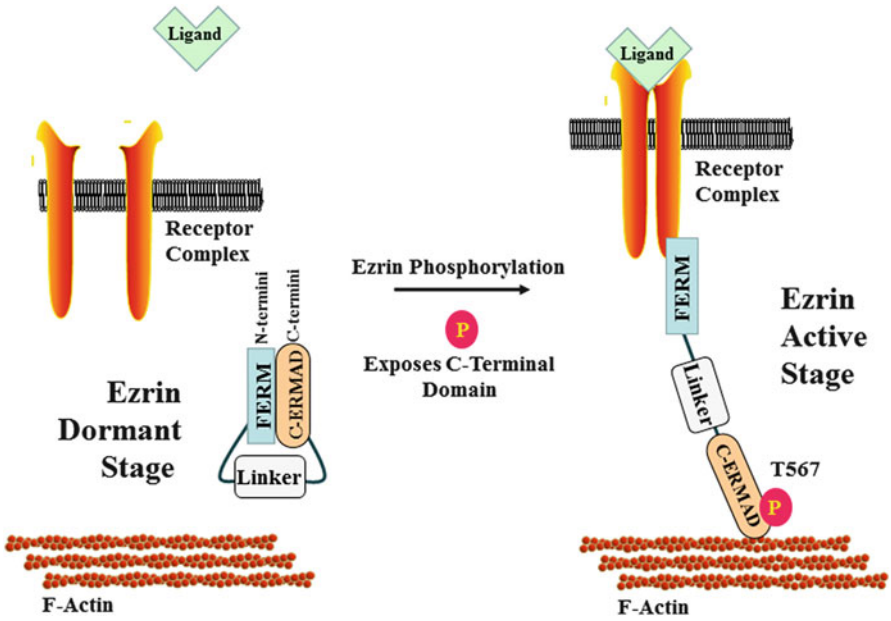

Fig. 2 Structural regulation of ezrin. Ezrin is normally in a dormant inactive stage with its N-terminal domain interacting and blocking the C-terminal domain. Ezrin is activated by phosphorylation. Ezrin threonine-567 is one of the most characteristic phosphorylation sites, and phosphorylation at threonine-567 causes the dissociation of the intramolecular interaction between the N- and C-terminal domains. This dissociation allows the N-terminal domain to interact with multiple receptor complexes and the C-terminal domain to interact with F-actin. *P* phosphorylation

3.1 EGFR

Ezrin is phosphorylated at tyrosine-145 in the N-terminus and tyrosine-353 in the central helical domain after the stimulation of *human* A431 epidermoid carcinoma cells with EGF (Hunter and Cooper 1981). These phosphorylations are associated with cellular differentiation and invasion (Saygideger-Kont et al. 2016; Bretscher 1989). Recent studies have shown that ezrin colocalized with EGFR, Na^+/H^+ exchanger 1 (NHE1), and β_1-integrin during invadopodia formation in tumor invasion and metastasis (Antelmi et al. 2013). The airway epithelial cells express high levels of EGFR during immune responses and cell remodeling in asthma and smoking. These results are consistent with multiple studies showing the potential regulation of EGFR signaling by ezrin (Ammar and Alice 2016; Jing et al. 2015; Burgel and Nadel 2008; Koff et al. 2008; Homma et al. 2015). The EGFR is activated by endothelin-1 in asthmatic airway smooth muscle cells and is involved in airway remodeling in asthma (Ammar and Alice 2016). EGFR also mediates the smoking-induced airway epithelium remodeling (Jing et al. 2015). These results suggest that ezrin contributes to the EGFR-induced modulation of airway cell remodeling and, thereby, respiratory disorders, such as asthma.

3.2 CD44 Receptor

Ezrin can also contribute to asthma by inducing CD44 de-polymerization (Lackie et al. 1997; Klagas et al. 2009; Casalino-Matsuda et al. 2009). CD44 is a transmembrane glycoprotein that is highly expressed on the surface of both immune and epithelial airway cells, and its expression is increased in the bronchial epithelium of asthmatic patients (Isacke and Yarwood 2002; Kumar et al. 2016; Lackie et al. 1997). However, CD44 expression is decreased in the airway smooth muscle cells of asthma and chronic obstructive pulmonary disease (COPD) patients, as shown by RT-PCR and Western blot analyses (Klagas et al. 2009). The de-polymerization of CD44 in the airway epithelial cells during inflammation also contributes to the hyper-secretion of mucus in asthma (Casalino-Matsuda et al. 2009). CD44 is also up-regulated in bronchial epithelial cells upon cellular damage in the airway, and the blockade of CD44 by neutralizing antibodies prevents cell migration (Leir et al. 2003). CD44 also interacts with other ERM (ezrin/radixin/moesin)-proteins (Yonemura et al. 1998). The GST-CD44 cytoplasmic domain binds to ERM-proteins with a high affinity, particularly to moesin, which has a K_D of $9.3 \pm 1.6 \times 10^{-9}$ M (a smaller equilibrium dissociation constant (K_D) indicates a higher affinity) (Hirao et al. 1996). Radixin binds to CD44 cytoplasmic peptides (292–363 residues) via the FERM domain, as demonstrated by structural studies (Mori et al. 2008). Because CD44 plays an important role in allergies, its interaction with ezrin has clinical implications as a potential pharmacological target (Katoh et al. 2011).

Similar to CD44, CD43 is a trans-membrane activation marker that interacts with ERM proteins and can contribute to asthma. CD43 regulates critical cellular functions, including T cell trafficking (Cannon et al. 2011). The activation of the T cell receptor (TCR) enhances the binding of ezrin to CD43, which induces the formation of a scaffold between the membrane and the cytoskeleton at the contact zone between the T cells and the antigen-presenting cells (APC) (Roumier et al. 2001). However, ezrin also colocalizes with CD43 in the opposite region, which is distal to the TCR engagement, suggesting that ezrin may contribute to the removal of inhibitory proteins from the immunological synapse during T cell activation (Allenspach et al. 2001).

3.3 VCAM

VCAM is expressed in tracheal smooth muscle and lung epithelial cells and modulates airway inflammation in asthma (Lin et al. 2015; da Silva et al. 2015). Immunoprecipitation assays have shown that VCAM-1 directly interacts with ezrin in the endothelial actin-rich docking structure, which mediates the leukocyte adhesion to the endothelium during inflammation in asthma (Barreiro et al. 2002). VCAM-1 signaling can be mediated by the advanced glycation end products (AGEs) receptor in pulmonary endothelial cells (Timothy et al. 2016). The N-terminal domain of ezrin binds to immobilized AGEs with a K_D value of $5.3 \pm 2.1 \times 10^{-7}$ M. These results suggest that this interaction is specific and likely mediated by the exposed ezrin N-terminal domain because neither the full-length nor the C-terminal domain binds to AGEs. The binding to AGEs inhibits ezrin phosphorylation and the subsequent formation of tubules in kidney LLC-PK1 cells (McRobert et al. 2003).

In addition to VCAM, ezrin interacts with other critical adhesion molecules in airway smooth muscle cells, such as intercellular cell adhesion molecule (ICAM) (Arij et al. 2015). Ezrin interacts with both ICAM-1 and ICAM-2 (but not with ICAM-3), and the ezrin–ICAM-2 interaction has a K_D of 3.3×10^{-7} M (Heiska et al. 1998). PIP_2 induces the interaction between ezrin and ICAM-1 and ICAM-2 (Heiska et al. 1998). ICAM-2 induces the phosphorylation of ezrin after the activation of Akt, which inhibits apoptosis in naive CD^{4+} cells (Perez et al. 2002). The crystal structures show that the ICAM-2 cytoplasmic domain binds to the groove of the phosphotyrosine binding (PTB)-like F3 subdomain of the N-terminal domain of radixin (Hamada et al. 2003).

3.4 DCC

The DCC is a part of the receptor complex of netrin-1 in the nervous system (Manhire-Heath et al. 2013). Netrin-1 regulates bleomycin-induced pulmonary