Co-occurrence of tetraspanin and ROS generators
Conservation in protein cross-linking and other developmental processes

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The nematode exoskeleton, commonly called the cuticle, is a highly structured extracellular matrix mainly composed of collagen. Secreted collagen molecules from the underlying epidermal cells are cross-linked via their tyrosyl residues. Reactive oxygen species (ROS) are required for the cross-linking reaction to produce tyrosyl radicals. The conserved ROS generator enzyme in C. elegans, BLI-3/CeDUOX1, a homolog of dual oxidases (DUOXs), is responsible for production of hydrogen peroxide. The ROS generation system must be properly controlled since ROS are highly reactive molecules that irreversibly inhibit the functions of cellular components such as nucleic acids and proteins. We recently reported that the ROS generation system directed by BLI-3 requires the tetraspanin protein, TSP-15. Herein we outline the process of cuticle development with a focus on the molecular roles of TSP-15 in the BLI-3 system. We also propose the co-occurrence of tetraspanin and ROS generators by convergent evolution.

Collagen Biosynthetic Pathway in Cuticle Development

Nematode cuticle possesses both toughness and flexibility, which protects internal tissues from adverse environments, maintains body morphology and mechanically supports locomotion via attachment to body wall muscle. Cuticle is predominantly composed of collagens encoded by over 170 genes in the C. elegans genome. Collagens are synthesized in underlying epidermal cells, the hypodermis and seam cells, covering almost the entire body. The collagen biosynthetic pathway is a multi-step processes involving modification, folding, cleavage and secretion directed by a number of enzymes and molecular chaperons.1 The importance of the co- and post-translational processing of collagen proteins has been supported by a comprehensive study of their mutants.2 Gain- or reduction/loss of function of the genes relevant to collagen processing are often associated with body morphological defects represented as dumpy (Dpy), roller (Rol), squat (Sqt), blister (Bli) and molting defect (Mlt). In the final events of processing, collagen triple helices are secreted from the epidermis and are extracellularly cross-linked. Each collagen helix is covalently cross-linked via tyrosine residues resulting in the formation of dityrosine and trityrosine, which are unusual non-reducible bonds in vertebrates (see below). In C. elegans, tyrosine cross-linking of collagens is established by ROS-mediated catalysis driven by the BLI-3/CeDUOX1 system.3-5 BLI-3 is a homolog of vertebrate NOX1–5 and DUOX1–2. These proteins are ROS generators that deliberately produce ROS for cellular signaling and anti-microbial responses.6,9

Identity and Difference of the Cross-Linking Process in Mammalian and Nematode Skin

Both nematode and mammalian skin layers share common structural...
characteristics, but mammalian skin is a more complex structure. It is composed of multilayered live and dead keratinocytes that constitute a cornified layer that faces the external side. Cross-linking of cytes that constitute a cornified layer that of multilayered live and dead keratinocytes is also essential for the function of mammalian skin as an external barrier. Transglutaminases (TGases) play a primary role in the cross-linking process, which catalyzes the formation of non-reducible covalent bonds between lysine and glutamate. Although tyrosine cross-linkages are found in other structural proteins at low frequency and are known as biomarkers of oxidative stress and aging, little is known about their physiological role in mammals. Hydroxyl lysine and lysine between collagen helices are cross-linked by lysyl oxidase in vertebrates. Lysyl oxidase-mediated cross-linkages were not found in the C. elegans cuticle, but they do contribute to cross-linking of type IV collagen in the basement membrane. Interestingly, both TGases- and ROS-mediated cross-linking is observed in the formation of the fertilization envelope in sea urchin egg.

**Tetraspanin is a New Component of the BLI-3/CEDUOX1-ROS-Generating System**

Historically, ROS have been considered deleterious by-products produced by aerobic metabolism or by exogenous stresses such as UV light and radiation, which inflict oxidative damage to organisms. The physiological role of ROS was originally believed to provide an “oxidative burst” that kills invading microbes in phagocytes. H$_2$O$_2$ produced by DUOXs also has an essential role in non-phagocytic anti-microbial defense in mucosal epithelia such as the airway and gastrointestinal tract in a wide-range of animals, including mammals, fish and insects. The critical role of H$_2$O$_2$ produced by BLI-3/CeDUOX1 in C. elegans innate immunity was also demonstrated. Besides host defense, ROS act as an intracellular redox signaling molecule by modulating target proteins via modification of their free thiol groups. In both cases, the ROS production must be strictly regulated so that it does not damage the host. The activity of the catalytic core of NOX1-3 is regulated by the recruitment of regulatory subunits to the plasma membrane. NOX5 and DUOX1-2 contains EF-hand motifs in the cytoplasmic region and calcium (Ca$^{2+}$) stimulation is essential for activation. In addition, DUOXs require interaction with their maturation factor, DUOXAs, for H$_2$O$_2$ production. Dual oxidase maturation factors (DUOXA1/2) dimerize with DUOXs to target DUOXs to the cell surface.

We previously reported that a tetraspanin protein TSP-15 is required for cuticle development for functioning as an external barrier. We recently clarified that TSP-15 functions in collagen cross-linking as a component of the DUOX system. Similar to mammalian DUOXs, the BLI-3 system also requires a maturation factor and cooperates with a neighboring heme peroxidase, which corresponds to DOXA-1 and MLT-7 in C. elegans, respectively. $^{4,30}$ bli-3, doxa-1 and mlt-7 mutants displayed the same cuticle deficiency as a tsp-15 mutant. Cuticle disorganization in the tsp-15 mutant is due to impaired tyrosine cross-linking during cuticle development. In addition, the tsp-15 mutant was restored by exogenous expression of both bli-3 and doxa-1, implying that these three genes are part of the same genetic pathway. We also showed requirement of TSP-15 for BLI-3 activity by heterologous reconstitution of BLI-3, TSP-15 and DOXA-1 in mammalian cells. Finally, we showed that TSP-15 forms protein complexes with BLI-3 and DOXA-1 in vitro and in vivo.

**Speculation of the Molecular Role of Tetraspanin in the BLI-3 System**

Despite our contributions to the field, the molecular role of TSP-15 in H$_2$O$_2$ generation by the BLI-3 system remains elusive. By immunoblot assay, TSP-15 did not alter the protein expression level of BLI-3 at the cell surface, leading us to question what molecular switch occurs within the BLI-3 system upon association with TSP-15. The tetraspanin family comprises a large group of integral membrane proteins with common secondary and tertiary structures, including four transmembrane regions, small and large extracellular loops (LEL) and conserved cysteine residues in the LEL contributing to the formation of disulfide bonds. It is known that tetraspanins laterally associate with each other as well as numerous membrane “partner” proteins such as adhesion molecules, growth factor receptors, membrane-bound proteases, immunoglobulin superfamily proteins. Tetraspanins also interact with intracellular signaling/cytoskeletal proteins and lipid, resulting in the formation of a highly ordered lipid-protein unit referred to as a tetraspanin-enriched microdomain (TEM/TERM) or “tetraspanin web.” TEM is a distinct class of membrane microdomain and a new type of signaling platform involved in cell-cell communication. Association with tetraspanins may properly tune functions of partner proteins. However, this modulation and facilitation process is not consistent – it may differ from partner to partner. The most likely role of TEM is in spatial assembly and clustering of specific molecules that contribute to accelerating the reaction cascade and enabling additional interactions and linkage with other key molecules and substrates. If the partner proteins are relevant to cell adhesion, antigen presentation or matrix degradation, it is convincible that the compartmentalization of these responsible molecules at specialized membrane microdomains will efficiently support their function in adhesion strengthening, cell-cell communication at immune synapses or ECM degradation in tumor invasion. It may also facilitate accessibility to substrates, while segregation of partner proteins to the microdomain may prevent non-specific reactions. We currently do not have enough information to provide mechanistic insights into the TSP-15 role in the BLI-3 system, however, we have several hypotheses. First, association with TSP-15 or targeting to TEM may support the subsequent recruitment of unknown factors, or it may facilitate other forms of post-translational modification on BLI-3 that is essential for BLI-3 activation. Or more directly, as reported for other NOX isozymes and their subunits, it might induce a conformational change in BLI-3 to activate it. Conversely, interaction with TSP-15 or engagement to TEM may...
Although it is still not known whether tetraspanin is also crucial for the mammalian DUOX pathway, conservation of involvement of tetraspanin in the ROS generation system is, at least in part, confirmed by genetic studies of pathogenic fungi. During the infection process of the rice pathogenic fungus *Magnaporthe grisea*, the attached conidium differentiates to appressorium, a specialized structure for infection. Then the appressorium develops a penetration peg and perforates the cell wall of the host tissues. It was independently demonstrated that the mutant of *M. grisea* tetraspanin (MgPLS1) and the ROS generator (MgNOX2) was non-pathogenic owing to impairment of penetration peg formation. The pathogenicity of other parasitic fungi with an appreciably mediated-penetration strategy in different clades was also dependent on PLS1 and NOX2. Both PLS1 and NOX2 were identified in other types of fungi with non-pathogenic lifestyles lacking appressorium. Furthermore, the consistency of the observed mutant phenotype was also consistent in different developmental processes in these saprophytic fungi. In *Podospora anserina* and *Neurospora crassa*, both of their corresponding mutants of PLS1 and NOX2 showed the same defects in the process of germination from the ascospore. Although the molecular mechanisms of requirement of PLS1 and NOX2 in these processes are still uncertain, the recurrent involvement of tetraspanin and ROS generators in the same cellular processes in a wide range of species makes it possible that convergent evolution is responsible for the co-occurrence of this molecular machinery.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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