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Claudin-31 contributes to corticosteroid-induced alterations in the barrier properties of the gill epithelium

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ABSTRACT

The contribution of Claudin-31 (Cldn-31) to corticosteroid-induced tightening of the trout gill epithelium was examined using a primary cultured model preparation. Cldn-31 is a ~23 kDa protein that localizes to the periphery of gill epithelial cells and diffusely in select gill cells that are Na^+-K^+-ATPase-immunoreactive. Transcriptional knockdown (KD) of *cldn-31* reduced Cldn-31 abundance and increased epithelium permeability. Under simulated *in vivo* conditions (apical freshwater), *cldn-31* KD increased net ion flux rates (≡ efflux). Cortisol treatment increased Cldn-31 abundance and decreased epithelium permeability. This tightening effect was diminished, but not eliminated, by *cldn-31* KD, most likely due to other cortisol-sensitive TJ proteins that were transcriptionally unperturbed or enhanced in cortisol-treated *cldn-31* KD preparations. However, *cldn-31* KD abolished a cortisol-induced increase in Cldn-8d abundance, which may contribute to compromised *cldn-31* KD epithelium permeability. Data suggest an important barrier function for Cldn-31 and an integral role for Cldn-31 in corticosteroid-induced gill epithelium tightening.

1. Introduction

The vertebrate tight junction (TJ) complex is an assembly of proteins found in the apicolateral domain of epithelial tissue where it forms connections between adjacent epithelial cells (Farquhar and Palade, 1963). TJ proteins contribute to the regulated passage of solutes across vertebrate epithelia and largely delineate the permeability properties of the paracellular pathway (reviewed by Günzel and Fromm, 2012; Günzel and Yu, 2013). The 'molecular backbone' of the TJ complex are claudin (Cldn) TJ proteins, which were first identified by Furuse et al. (1998a,b). Cldn proteins are tetraspanins, each one of which possesses two extracellular loops (ECLs). Cldn ECLs from adjacent epithelial cells interact in the intercellular cleft to form an occluding barrier. However, occlusion is not absolute as the molecular properties of Cldn TJ protein ECLs permit the regulated movement of specific solutes based on size and/or charge. In this regard, it is the constituent amino acids of Cldn ECLs, and in particular the first ECL, that establish the perm-selectivity properties of the paracellular pathway (Colegio et al., 2003).

Cldn TJ proteins exhibit very distinct expression patterns in vertebrate epithelia and this is thought to underpin the diverse permeability characteristics of epithelial tissue in the vertebrate clade (reviewed by Günzel and Fromm, 2012; Günzel and Yu, 2013). In the gill tissue of fishes, at least 44 genes encoding Cldn TJ proteins have been described to date from a relatively small number of teleosts (Kolosov et al., 2013), and based on the keystone study of Loh et al. (2004) that characterized the Cldn superfamily of *Fugu rubripes*, any given species of teleost fish will likely express ~ 32 *cldns* in the gill. Current evidence suggests that some of these proteins are found in both the gill epithelium and vasculature, while other proteins are found only in specific cell types within the gill epithelium (Bui et al., 2010; Bui and Kelly, 2014; Gauberg et al., 2016; Kolosov et al., 2014). In addition, using cultured gill epithelium models derived from either freshwater (FW) or seawater (SW) fishes, it has also been established that alterations in the transcript and/or protein abundance of select TJ proteins occur in association with changes in the flux rates of paracellular permeability markers as well as ions such as Na^+ and Cl^- (Bui and Kelly, 2015; Chasiotis et al., 2010, 2012a; Sandbichler et al., 2011). Nevertheless, although we are beginning to gain insight into what role select TJ proteins might play in the gill epithelium (reviewed by Chasiotis et al., 2012b), mechanistic studies that target specific Cldn TJ proteins in order to delineate their contribution to gill epithelium

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barrier properties are missing.

Corticosteroids have a well-established tightening effect on diverse vertebrate epithelia and endothelia (e.g. Burek and Förster, 2009; Casey and Plaut, 2007; Felinski et al., 2008; Förster et al., 2008; Kashiwamura et al., 2011; Kimura et al., 2011; Stelwagen et al., 1999; Zettl et al., 1992). In fishes, this direct effect of corticosteroids on gill epithelium permeability was first observed using a primary cultured gill model derived from FW rainbow trout (Kelly and Wood, 2001). In this model, cortisol was observed to dose-dependently increase TER and decrease the flux rate of a paracellular permeability marker, as well as decrease basolateral to apical Na^+ and Cl^- flux rates (≡ to a reduction in diffusive ion loss). These changes were proposed to be beneficial to a stressed FW fish (within which circulating cortisol levels would be elevated) by helping to mitigate ion loss. Since then, the same tightening effect of corticosteroids has been observed in gill epithelium models derived from other species of fish (Kelly and Wood, 2002; Chasiotis and Kelly, 2011) as well as in larval zebrafish (Kwong and Perry, 2013). Furthermore, recent studies have established a causal relationship between corticosteroid-induced epithelial tightening in the gill epithelium of teleost fishes and alterations in the transcript and/or protein abundance of select Cldn TJ proteins as well as other tetraspanin transmembrane TJ proteins such as occludin and tricellulin (Bui et al., 2010; Chasiotis and Kelly, 2011, 2012; Chasiotis et al., 2010; Kelly and Chasiotis, 2011; Kolosov and Kelly, 2013). This response of TJ proteins to cortisol in the fish gill epithelium presents an opportunity to investigate how TJ proteins contribute to corticosteroid-mediated changes in the barrier function of a unique tissue that interfaces with water on one side and extracellular fluid on the other. At the same time, using cortisol to manipulate the contribution of select 'cortisol-responsive' TJ proteins will allow further insight into the contribution of specific TJ proteins to the barrier properties of the gill.

At the transcriptional level, Cldn-31 is a corticosteroid-responsive TJ protein in the gill epithelium of rainbow trout, as its mRNA abundance has been observed to increase significantly both in the presence of cortisol as well as the glucocorticoid agonist dexamethasone (Kelly and Chasiotis, 2011). Given that these changes occurred in a cultured gill epithelium composed of gill pavement cells (PVCs) only, and in association with a corticosteroid-induced reduction in epithelium paracellular permeability, there is reason to believe that Cldn-31 is a barrier protein in the gill epithelium. In addition, there are other lines of evidence suggesting that Cldn-31 plays an important barrier-forming role in the trout gill epithelium. First, transcript abundance of *cldn-31* is intermittently upregulated in a cultured trout gill epithelium during the development of resistive properties (Kolosov et al., 2014). Secondly, *cldn-31* mRNA is increased in the gill of rainbow trout exposed to ion-poor water (IPW), which is an environment that requires enhanced gill barrier properties to moderate amplified diffusive ion loss (Perry and Laurent, 1989; Kolosov and Kelly, 2016; Chen et al., 2016). However, if the IPW challenge was preceded with dietary NaCl loading, which reduces the need to mitigate ion loss, no alteration in *cldn-31* was observed (Kolosov and Kelly, 2016). Of additional interest is that *cldn-31* transcript abundance has been reported to increase 60-fold in a cultured trout gill epithelium that contains mitochondrion-rich cells versus those that contain PVCs only (Kolosov et al., 2014). Thus, Cldn-31 may be enriched in mitochondrion-rich gill ionocytes, suggesting that its contribution to gill epithelium barrier properties may differ by cell type.

Taking the above observations together, it can be hypothesize that Cldn-31 is a barrier protein in the gill epithelium of fishes. Furthermore, it can also be hypothesized that Cldn-31 plays a role in corticosteroid-induced alterations in gill epithelium permeability. However, what is unclear for Cldn-31, and has yet to be determined for any Cldn, is that if it does contribute to the barrier properties of the gill and/or corticosteroid-induced tightening of this epithelium, how much does it contribute? Therefore, the objective of the current study was to gain further insight into the molecular physiology of Cldn-31 and its role in corticosteroid-induced gill epithelium tightening.

2. Materials and methods

2.1. Experimental animals

Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) were purchased from Humber Springs Trout Hatchery (Orangeville ON, Canada), transported to the lab and held in 600 L opaque polyethylene tanks supplied with flow-through dechlorinated freshwater (FW, composition in μM: [Na^+] 590, [Cl^-] 920, [Ca^{2+}] 760, [K^+] 43, pH 7.35). Water temperature ranged between 8 and 10 °C and the photoperiod was a constant 12 h light:12 h dark cycle. Fish were fed *ad libitum* once daily with commercial trout pellets (Martin Profishent, Elmira, ON, Canada). Animal husbandry and experimental procedures were conducted in accordance with an approved York University Animal Care Committee protocol which conformed to the guidelines of the Canadian Council on Animal Care.

2.2. Characterization of rainbow trout Cldn-31

TOPO2 software was used to visualize protein sequence of Cldn-31 translated from full coding mRNA (GenBank BK007969). Hydrophobicity and transmembrane domain tendency was predicted using ProtScale and TMHMM online engines. Based on sequence information for rainbow trout Cldn-31, a custom synthesized polyclonal antibody was raised in rabbit against a synthetic peptide (Ac-CFSSDSSPRRGPAAQ-amide) corresponding to the 196–210 amino acid region of rainbow trout Cldn-31 (GenScript, Piscataway, NJ, USA). Western blot analysis of Cldn-31 was conducted using methods previously described by Chasiotis et al. (2010). Briefly, insert-cultured cells were rinsed with ice-cold PBS (4 °C) then incubated in a lysis buffer (10 mM Tris-HCl, pH = 7.5, 1 mM EDTA, 0.1 mM NaCl, 1 mM PMSF) with 1:200 protease inhibitor cocktail (Sigma-Aldrich Canada Co., Oakville, ON, Canada). Epithelial tissue was then collected after homogenization by repeatedly passing cells in solution through a 26G syringe needle. A supernatant was collected following centrifugation of homogenized samples at 10,000 g for 10 min at 4 °C and protein concentration of this solution was determined using a standard Bradford Assay (Sigma-Aldrich Canada Co.). A total of 5 μg protein was then used for Western blotting using a 12% SDS-PAGE gel. Wet transfer was performed to transfer protein samples from the gel to a polyvinylidene difluoride (PVDF) membrane at 100 V for 1 h. Following transfer, the membrane was incubated with 5% skimmed milk solution for 1 h at room temperature (RT), after which the membrane was incubated with the rabbit anti-Cldn-31 custom synthesized antibody (1:1000 dilution in Tris-buffered saline with Tween (TBS-T, 10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH = 7.4) overnight at RT with constant agitation. Signal detection was performed by incubating the membrane with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Bio-Rad Laboratories Canada Ltd.) for one hour at RT. Antigen reactivity was then examined by incubating the membrane with Clarity™ Western ECL Blotting Substrate (Bio-Rad Laboratories Canada Ltd.) for 5 min at RT. Imaging of immunoreactivity was conducted using a Chemi-Doc™ MP System (Bio-Rad Laboratories Canada Ltd.). Specificity of the custom-made primary antibody was confirmed using a peptide