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Sound and acceleration are detected by hair bundles, mechanosensitive structures located at the apical pole of hair cells in the inner ear. The different elements of the hair bundle, the stereocilia and a kinocilium, are interconnected by a variety of link types. One of these links, the tip link, connects the top of a shorter stereocilium with the lateral membrane of an adjacent taller stereocilium and may gate the mechanotransducer channel of the hair cell. Mass spectrometric and Western blot analyses identify the tip-link antigen, a hitherto unidentified antigen specifically associated with the tip and kinocilial links of sensory hair bundles in the inner ear and the ciliary calyx of photoreceptors in the eye, as an avian ortholog of human protocadherin-15, a product of the gene for the deaf/blindness Usher syndrome type 1F/DFNB23 locus. Multiple protocadherin-15 transcripts are shown to be expressed in the mouse inner ear, and these define four major isoform classes, two with entirely novel, previously unidentified cytoplasmic domains. Antibodies to the three cytoplasmic domain-containing isoform classes reveal that each has a different spatiotemporal expression pattern in the developing and mature inner ear. Two isoforms are distributed in a manner compatible for association with the tip-link complex. An isoform located at the tips of stereocilia is sensitive to calcium chelation and proteolysis with subtilisin and reappears at the tips of stereocilia as transduction recover after the removal of calcium chelators. Protocadherin-15 is therefore associated with the tip-link complex and may be an integral component of this structure and/or required for its formation.

Key words: protocadherin-15; tip link; TLA; stereocilia; mechanotransduction; hair cell

Introduction

Hearing and balance depend on hair cells, polarized epithelial cells of the inner ear that have a mechanosensitive hair bundle located at their apical pole. The hair bundle is composed of numerous stereocilia and, in all organs except the mature cochlea, a single kinocilium. These elements are coupled to one another by a variety of links. Up to four distinct interstereocilial link types, tip links, horizontal top connectors, shaft connectors, and ankle links, can be distinguished in avian hair bundles (Goodyear and Richardson, 1992). A fifth link type, the kinocilial link, connects the kinocilium, when present, to the adjacent stereocilia (Goodyear and Richardson, 2003). The tip link, a slender filament connecting the top of one stereocilium to the side of an adjacent taller stereocilium, is a feature of all hair bundles and is thought to gate the mechanotransducer channel of the hair cell (Pickles et al., 1984; Assad et al., 1991). The other links, which can vary in their distribution according to species, age, and hair-bundle type, may hold stereocilia together as a coherent unit and transmit forces across the bundle.

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ankle-link antigen (ALA) with ankle links (Goodyear and Richardson, 1999), and the tip-link antigen (TLA) with tip and kinocilial links (Goodyear and Richardson, 2003). Some of these antigens and other candidate link components have been recently identified. Cadherin-23 is a component of the kinocilial links and transient lateral links of immature hair bundles (Lagzi et al., 2005; Michel et al., 2005) and a candidate for a component of the tip link in mature hair cells (Siemens et al., 2004). The HCA is the receptor-like inositol lipid phosphatase Ptpq (Goodyear et al., 2003), and the ALA is the very large G-protein-coupled receptor Vgr1 (Goodyear et al., 2006). Usherin and protocadherin-15 (Ahmed et al., 2001; Alagramam et al., 2001a) may also be associated with ankle links (Adato et al., 2005; Senften et al., 2006). Protocadherin-15 is a member of the cadherin superfamily of Ca2+-dependent adhesion glycoproteins, and its importance for the normal architecture of the hair cell stereocilia bundle and inner ear function is well established in humans, mice, and zebrasfish (Ahmed et al., 2001, 2003; Alagramam et al., 2001a,b; Ben-Yosef et al., 2003; Seiler et al., 2005).

In this study, we identify the TLA by mass spectrometry peptide sequencing as an epitope of protocadherin-15. Furthermore, we reveal that novel isoforms classes of protocadherin-15 are expressed in the mouse and chicken inner ear and that these can be defined by the absence or presence of one of three alternative unique cytoplasmic domains. Three of these isoforms classes are shown to have dramatically different spatiotemporal expression patterns within the developing and mature hair bundle, and two are distributed in a manner that allows association with the tip-link complex.

**Materials and Methods**

cDNA cloning and sequence analysis. Full-length mouse Pdcdh15 poly(A)+ RNA was isolated from postnatal day 1 (P1) to P5 inner ear tissue dissected from 50 C57BL/6J mice using Poly(A)Pure (Ambion, Austin, TX). cDNA was prepared using an oligo-dT primer and PowerScript reverse transcriptase (Clontech, Cambridge, UK). PCDH15 transcripts were amplified from human retina cDNA (GETRare; Genemmed Synthesis, South San Francisco, CA). To determine the structure and isoforms of chicken Pdcdh15, we used poly(A+) RNA from chicken brain (Stratagene, La Jolla, CA). Supplemental Table S1 (available at www.jneurosci.org as supplemental material) provides the sequences of the primers used to amplify PCDH15/Pdcdh15 from human, mouse, and chicken tissues. All PCR products were subcloned, and all strands were fully sequenced.

Antibodies. Mouse mAb G19 directed against the TLA from chicken inner ear hair cells, mAb D10 directed against the avian hair-cell antigen, and antisera to protocadherin-15-CD1 (PB303) were characterized and validated as reported previously (Richardson et al., 1990; Ahmed et al., 2003; Goodyear and Richardson, 2003). Additional peptides based on mouse protocadherin-15 (shown in Fig. 1) were synthesized by Prince- ton BioMolecules (Langhorn, PA) and used to immunize New Zealand white rabbits (Covance Research Products, Welwyn Garden City, CA) or anti-His (Roche Products) monoclonal antibodies for 2 h. Cells were rinsed three times in PBS and were incubated with anti-Flag (Roche Products, Welwyn Garden City, CA) or anti-His (Roche Products) monoclonal antibodies for 2 h. The specificity of antibodies raised against different epitopes of protocadherin-15 was validated using inner ear tissue from homozygous av-3f mice and by blocking assays performed with transiently transfected cell lines. A Flag-tagged sequence encoding the C-terminal 194 amino acids of chicken protocadherin-15–CD1 was amplified by reverse transcription (RT)-PCR with Pfu polymerase (Stratagene) and primers GGCdcdh15F2 (gcagcattgctttctccctaccacaTCT) and GPCdcdh15R1 (cacgccagtcacggtgcgttagaggt) from total RNA isolated from 2 d posthatch chick utricle. The PCR product was digested with Ndel and BamH1 and ligated into the same sites of pET15b (Novagen, Nottingham, UK). The 6His-tagged fusion protein was expressed in E. coli BL21 (DE3) pLysS and purified by nickel affinity column chromatography. The purified fusion protein was used to immunize a CD1 mouse, and the immune serum obtained from a tail bleed was used at a dilution of 1:1000 for immunoblotting.

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For antisera blocking experiments, anti-protocadherin-15 antisera were preabsorbed for 30 min with a 10-fold excess concentration (over that of affinity-purified antisera) of the corresponding expressed protein or peptide(s) and then incubated with transfected cells for 2 h. Cells were rinsed three times in PBS and were incubated with anti-Flag (Roche Products, Welwyn Garden City, CA) or anti-His (Roche Products) monoclonal antibodies for 2 h. After three rinses in PBS, samples were incubated in a 1:200 dilution of the FITC-conjugated anti-rabbit IgG secondary antibody (Amersham Biosciences) and 1:200 dilution of the Alexa 568-conjugated anti-mouse IgG secondary antibody (Invitrogen) for 30 min. Samples were washed three times with PBS and mounted using a ProLong Antifade kit (Invitrogen) and viewed in an LSM510 Zeiss (Oberkochen, Germany) confocal microscope. There are five identical amino acid residues (DYLLR) in the cytoplasmic domains of cadherin 23 and protocadherin-15–CD2. We tested our protocadherin-15 antibodies for cross-reactivity with cadherin-23 and found none.

**Immunocytochemistry.** Immunocytochemistry was performed as described previously (Belyantseva et al., 2005; Michel et al., 2005). For labeling experiments involving cochlear cultures and whole mounts, 4 to 12 explants or maculae were examined for each condition.

**Immunofluorescence purification of TLA.** mAb G19 recognizes an extracellular epitope associated with the tip link (Goodyear and Richardson, 2003). An affinity column was prepared commercially (Immune Sys-
tems, Bristol, UK) by conjugating 11 mg of mAb G19 that had been affinity purified from hybridoma supernatant on Protein A to 3 ml of Sepharose CL-4B resin. Retinas were dissected from 180 2-d posthatch chicken eyes in PBS containing protease inhibitors (1 mM PMSF, 2 mM benzamidine, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) and frozen. The frozen retinas were thawed and homogenized in 50 ml of extraction buffer (150 mM NaCl, 1% TX-100, 5 mM CaCl₂, and 20 mM HEPES, pH 7.2) containing EDTA-free protease inhibitor cocktail (Roche Products) and centrifuged at 41,000 × g for 30 min. The resultant supernatant was filtered through Whatman (Maidstone, UK) #1 filter paper, recentrifuged at 48,000 × g for 30 min, and passed three times through the mAb G19 affinity column. The column was washed sequentially with 40 ml of extraction buffer, 60 ml of high-salt wash buffer (0.5 M NaCl, 0.1% TX-100, 5 mM CaCl₂, and 20 mM HEPES, pH 7.2), and 10 ml of 1 M MgCl₂ (in 0.1% TX-100, 5 mM CaCl₂, and 20 mM HEPES, pH 7.2), and finally eluted with 6 ml of glycine-HCl, pH 2.0. The glycine-HCl eluate was neutralized with Tris base, dialyzed extensively against several changes of water, and lyophilized. The lyophilized sample was resuspended in reducing SDS-PAGE sample buffer, heated at 100°C for 4 min, and separated on a 6% polyacrylamide gel. Coomassie-stained protein bands of ~250 and 200 kDa corresponding to the previously identified TLA were excised and washed extensively in water.

Figure 1. Pcdh15 splice variants. Splicing of the primary transcripts of Pcdh15 and the four isoform classes defined by the presence or absence of one of three different cytoplasmic domains. Newly discovered exons of Pcdh15 are designated with a letter suffix if located among the reported 35 exons. A dashed line indicates that one or more exons were not included in the transcript. A dotted line designates either the 3′ untranslated region (UTR) or the 5′ UTR. A signal peptide is encoded by exon 2, and a transmembrane domain (TM; brown) is encoded by exon 31.

a–c, Diagrams of exon content of full-length, open reading frame-containing transcripts with one of the three alternative cytoplasmic domains encoded by exons 35 (green), 38 (blue), and 39 (pink), respectively (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Different antisera used for producing polyclonal antibodies to protocadherin-15 are shown as yellow rectangles above and below the structures of each protocadherin-15 isoform. The location of the two tryptic chicken peptides identical to human and mouse sequence that were detected by mass spectrometric analyses of purified TLA are shown as two red rectangles below the structure of protocadherin-15–CD1 (for sequence of peptides, see Table 1). The locations of the six reported Ames waltzer alleles (av-2J, av-Tg2742Rpw, av-5J, av-Jfb, av-J, and av-3J) are shown in a. Note that, for all transcripts of mouse Pcdh15 shown in a–c, there are no constant coding exons. However, within each class of Pcdh15 transcripts (CD1, CD2, and CD3), the 3′ UTR encoded by exons 35, 38, and 39, respectively, appear to be constant, but that observation may reflect an ascertainment bias because the reverse primer for each of the three classes was located in the 3′ UTRs (green, blue, and pink arrows) (see also Ahmed et al., 2003). Black arrow in exon 1 is the location of the forward primer. d, An isoform class of protecadherin-15 expressed in the mouse inner ear that is predicted to be secreted. The GenBank accession number for each isoform is in supplemental Table S3 (available at www.jneurosci.org as supplemental material).
Figure 2. Distributions of protocadherin-15–CD1, protocadherin-15–CD2, and protocadherin-15–CD3 in the developing mouse cochlea. P2 mouse organ of Corti, 1 d in vitro. Hair bundles from the apical end of the apical coil (a, d, g), middle of the apical coil (b, e, h), and basal (c, f, i) regions of the cochlea stained with antibody PB303 to protocadherin-15–CD1 (a–c), antibody PB464-2B to protocadherin-15–CD2 (d–f), and antibody PB375 to protocadherin-15–CD3 (g–i). The left half of each panel shows the distribution of protocadherin-15, and the right half shows the merge with F-actin. I, Inner hair cell; O1, O2, O3, outer hair cells in rows 1, 2, and 3 respectively. Scale bars, 10 μm.

Mass spectrometric analysis of TLA. Acrylamide gel slices containing TLA were reduced, carboxymethylated, digested to peptides using trypsin on a MassPrepStation (Waters, Manchester, UK), desalted, and concentrated. To obtain peptide mass and sequence, the resulting peptides were applied to a capillary liquid chromatography (LC) column coupled to a quadrupole tandem time-of-flight mass spectrometer (LC-MS/MS) (QToF2; Waters). For LC-MS/MS, a reverse-phase liquid chromatography system (Waters) attached to the QToF2 mass spectrometer. The MS/MS data obtained from the two TLA protein bands (see Fig. 7a, bands 1, 2) were then used to search the National Center for Biotechnology Information (NCBI) human and mouse databases using the Mascot search engine. Probability-based Mascot scores were used to evaluate identifications. Only matches with p < 0.05 for random occurrence were considered significant. Because the TLA was affinity purified from the chicken, the same data files were submitted to the search software BioWorks (Thermo Electron Corporation, Waltham, MA). The chicken protein database was downloaded directly from the NCBI, to which chicken protocadherin-15–CD1, protocadherin-15–CD3, and protocadherin-15–CD2/CD3 amino acid sequences were added by us and are now available in the chicken database (supplemental Table S3, available at www.jneurosci.org as supplemental material).

Immunoblot analysis. The TLA was immunoprecipitated from retinal extracts with minor modifications of the method described previously (Goodyear and Richardson, 2003). Retinas were homogenized in extraction buffer containing 1% Triton X-100 and the protease inhibitors PMSF (1 mM), benzamidine (2 mM), pepstatin (1 μg/ml), and leupeptin (1 μg/ml), centrifuged at 20,000 × gmax for 20 min at 4°C. The supernatant was recentrifuged at 4000 × gmax for 10 min, divided into two aliquots, and mixed overnight at 4°C with goat anti-mouse IgG1, Fc agrose beads that had been preloaded with either mAb G19 anti-TLA or mAb D10 anti-hair cell antigen (Richardson et al., 1990) hybridoma supernatants. mAb D10 is an mAb of the same subclass (IgG1) as G19 that specifically recognizes sensory hair bundles in the inner ear and does not stain retina. The beads were collected by sedimentation at 1 × g and washed six times with PBS/0.1% Triton X-100, and bound proteins were eluted by heating to 100°C for 4 min in SDS-PAGE sample buffer. Eluted proteins were resolved on 6% SDS polyacrylamide gels and transferred to HybondP using semidry blotting. The blots were preblocked in TBS containing 0.05% Tween-20 and 3% low-fat milk powder, incubated overnight in preblocking solution containing polyclonal antibodies PB473-3, PB303, or HIL5383 at 0.5 μg/ml or polyclonal mouse anti-chick protocadherin-15–CD1 intracellular domain serum (M110) at a dilution of 1:2000, washed, and reacted with alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse IgG (both at 1:1000 dilution; Dako, High Wycombe, UK) for 2 h. Bound antibodies were visualized with nitroblue-tetrazolium-chloride/5-bromo-4-chlor-indolyl-phosphate (Roche Diagnostics, Lewes, UK).

immunogold electron microscopy. Utricular maculae were rapidly dissected from the inner ear of P2 CD1 mice in HEPES-buffered (10 mM, pH 7.2) HBSS (HBHBBS) and fixed for 1 h in 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4. For pre-embedding labeling with PB375, the samples were washed in PBS, preblocked and permeabilized in TBS containing 0.1% Triton X-100 for 1 h, and incubated overnight at 4°C in the same solution containing affinity-purified PB375 or non-immune rabbit IgG at a concentration of 10 μg/ml. After extensive washing, the samples were incubated overnight in goat anti-rabbit IgG conjugated to 5 nm colloidal gold (British Biocell International, Cardiff, UK) at a dilution of 1:100, washed, re-fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate containing 0.5% ruthenium red, washed in buffer, and postfixed in 1% OsO4. Sections were dehydrated in ethanol, embedded in Epon, and sectioned at a thickness of 100 or 200 nm. Chicken utricular maculae were double labeled with mAb G19 and rabbit anti-PB473-3 using a similar protocol except that a mixture of goat anti-mouse and goat anti-rabbit IgGs conjugated, respectively, to 10 and 5 nm colloidal gold were used to detect the primary antibodies. Controls with just one primary antibody (G19 or PB473-3) and both gold conjugates were used to validate the specificity of labeling. For postembedding labeling, paraformaldehyde-fixed mouse maculae were dehydrated with cold ethanol and infiltrated with cold Unicryl resin overnight. The maculae were placed in BEEM capsule caps with fresh Unicryl resin, the resin-filled caps were covered with a plastic coverslip, and the resin was polymerized in the cold by exposure to UV light. Thin sections were cut and stained with PB375, PB303, or non-immune rabbit IgG at a concentration of 10 μg/ml followed by goat anti-rabbit IgG conjugated to 10 nm colloidal gold as described previously (Thorpe, 1999). Sections were counterstained with unryl acetate and lead citrate and viewed in a Hitachi (Wokingham, UK) 71000 microscope operating at 75 or 100 kV, and images were captured with a Gatan (Abington, UK) Ultrascan 1000 CCD camera. For pre-embedding labeling, a minimum of four tissue blocks were sampled at several levels for each condition, and, for postembedding labeling, a minimum of 20 sections were examined with each antibody.

Treatment of cochlear cultures with BAPTA and subtilisin. Cochlear...
cultures were prepared from the inner ears of P1–P2 mouse pups on collagen-coated round glass coverslips as described previously (Russell and Richardson, 1987) and grown overnight in Maximow slide assemblies in a medium containing 93% DMEM/F-12, 7% fetal calf serum, and 10 μg/ml ampicillin. Cultures were removed from the Maximow slides, placed in 35-mm-diameter plastic Petri dishes, and washed once with 3 ml of HBHBSS (for subsequent saline or subtilisin treatment) or 3 ml of Ca²⁺ free HBHBSS (for subsequent BAPTA treatment). Cultures were then treated with HBHBSS, HBHBSS containing 50 μg/ml subtilisin (Protease XIV; Sigma, Poole, UK), or Ca²⁺ free HBHBSS containing 5 mM BAPTA for 15 min at room temperature before fixation in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. To examine the recovery of protocadherin-15 epitopes, the BAPTA treatment time was reduced to 5 min, and cultures were washed three times over a 5 min period in a large volume of HBHBSS and then fixed or replaced in Maximow slides with medium and cultured for an additional 1, 4, or 24 h at 37°C.

Results
Novel protocadherin-15 isoforms are expressed in the inner ear
Regions of conserved DNA sequence with open reading frames are located downstream from the exons encoding the previously reported (Ahmed et al., 2001) C-terminal cytoplasmic domain (CD1) of protocadherin-15 in the human, mouse, rat, chicken, and zebrafish. RT-PCR analyses reveal transcripts in the mouse inner ear and the human retina that have a subset of additional alternatively spliced exons encoding two novel cytoplasmic domains (CD2 and CD3). These alternatively spliced Pcdh15 transcripts use a subset of the exons encoding the signal sequence, the extracellular domain (up to 11 extracellular cadherin repeats), a single-pass transmembrane domain, and sequence encoding cytoplasmic domain CD1, CD2, or CD3 (Fig. 1a–c).

Each of the three cytoplasmic domain classes has a unique amino acid sequence (542, 379, and 319 residues, respectively). CD1, CD2, and CD3 also have unique C termini (STSL, NTAL, and MTKL, respectively) (Fig. 1) (supplemental Fig. S1a–c, available at www.jneurosci.org as supplemental material), which are type 1 PDZ (postsynaptic density 95/Discs large/zona occludens-1)-binding consensus sequences (X-T-X-L) (Sheng and Sala, 2001). Among 10 species for which the different cytoplasmic domain cassettes (CD1, CD2, and CD3) of protocadherin-15 were compared, the amino acid sequence of CD3 is the most highly conserved (data not shown).

Surprisingly, the three cytoplasmic domains of protocadherin-15 (CD1, CD2, and CD3) (Fig. 1) do not comprise an exhaustive list. In humans and mice, we also discovered the identical composites of alternatively spliced portions of exons encoding either CD1 and CD3, or CD1, CD2, and CD3 (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). In addition, a class of transcripts of Pdhl15 (protocadherin-15-Sld) is expressed in the mouse inner ear that lacks the exons encoding the transmembrane and cytoplasmic domains and is likely to produce secreted isoforms of protocadherin-15 (Fig. 1d) (Rouget-Quermalet et al., 2006).

Protocadherin-15 isoforms have different spatiotemporal expression patterns
The distribution of the different isoform classes in the hair bundles of the developing inner ears of rats and mice was examined using antibodies generated against peptide sequences unique to each isoform class. The specificity of each antiserum for protocadherin-15 was verified by an absence of staining in the
Figure 4. Protocadherin-15–CD3 distribution in hair bundles. a–g. Confocal images of hair bundles double labeled with antibodies to protocadherin-15–CD3 (PB375 or HL5383; green) and phalloidin (red). In a–e, the left or top panel shows the distribution of protocadherin-15–CD3, the middle panel shows the distribution of F-actin, and the right or bottom panel shows the merge of the two labels. In f and g, the left panels shows protocadherin-15–CD3, the middle panel shows the distribution of F-actin, and the right or bottom panel shows the merge of the two labels. In f and g, the left panels shows protocadherin-15–CD3, the middle panel shows the distribution of F-actin, and the right or bottom panel shows the merge of the two labels. Scale bars: a, b, d–g, 5 μm; c, 1 μm.

Hair bundles of homozygous av-3J mice (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). In cell lines transfected with either an epitope-tagged expression construct of protocadherin-15–CD1 (the last five ECs were deleted) or epitope-tagged forms of the protocadherin-15 cytoplasmic domains CD1, CD2, and CD3, the staining pattern observed with each antisera was abolished by preincubating each serum with the peptide to which it was raised but not by a mixture of the peptides specific for the other two isoforms (supplemental Fig. S3a–f, available at www.jneurosci.org as supplemental material).

In the cochlea, hair cells in the basal, high-frequency end of the duct differentiate before those in the apical, low-frequency region, and, at any one stage of embryonic or early postnatal development, a gradient of hair-bundle maturation is seen along the length of the cochlea. In cochlear cultures at the equivalent of postnatal day 3, clearly opposing gradients of staining are observed with antibodies specific for CD1 (Fig. 2a–c) and CD2 (Fig. 2d–f). Antibodies to CD1 do not stain hair bundles at the apical end of the apical coil (Fig. 2a), stain those in the middle of the apical coil (Fig. 2b), and react intensely with those in the basal coils (Fig. 2c). Antibodies to CD2 stain hair bundles in the apex of the cochlea intensely at this stage of development (Fig. 2d), those in the middle of the apical coil stain weakly (Fig. 2e), and, at the base of the cochlea, staining is barely detectable (Fig. 2f). With antibodies specific for CD3, a gradient of hair-bundle staining is observed that is similar to that seen with antibodies to CD1, i.e., little or no staining is seen at the apex (Fig. 2g), moderate staining is seen in the middle (Fig. 2h), and strong staining is seen in the base (Fig. 2i).

In mature hair bundles, protocadherin-15–CD1 is distributed fairly evenly along most of the length of the stereocilia on auditory hair cells (Fig. 3a), whereas it is concentrated toward the upper third of the hair bundle in vestibular hair cells (Fig. 3b). In both the auditory and the vestibular organs, protocadherin-15–CD1 is excluded from a region at the very tip of each stereocilium (Fig. 3c,d). Protocadherin-15–CD2 immunoreactivity decreases to an undetectable level in adult mouse cochlear hair cells (data not shown). Immature and mature hair cells are both present in the sensory epithelium of the early postnatal rodent vestibule. The entire hair bundle of the immature cells stains uniformly and intensely for protocadherin-15–CD2, whereas only the kinocilium is stained in the more mature hair bundles (supplemental Fig. S4, available at www.jneurosci.org as supplemental material).

With antibody PB375 directed against a unique peptide sequence in protocadherin-15–CD3, staining is detected in immature vestibular hair bundles as early as embryonic day 15.5 (E15.5) and is concentrated toward the tip of each stereocilium (Fig. 4a). Protocadherin-15–CD3 also localizes to the tips of the
shorter stereocilia in the mature vestibular hair bundles of adult mice and rats and is not detected at the tips of the stereocilia in the tallest row (Fig. 4b,c). In the more mature hair bundles at the base of the cultured mouse cochlea at the equivalent of P3, staining with PB375 is restricted to the tips of the shorter stereocilia in both inner (Fig. 4d) and outer (Fig. 4e) hair cells. A similar staining pattern is observed in hair bundles throughout most of the length of the cochlea at this stage but not in those of outer hair cells at the very apical end of the sensory epithelium (Fig. 2g). Protocadherin-15–CD3 immunoreactivity (PB375) is not detectable in the hair bundles of the mature cochlea using these antibodies (data not shown). An affinity-purified antibody (HL5383) directed against a GST–CD3 fusion protein (validated as described above) (supplemental Figs. S2, S3, available at www.jneurosci.org as supplemental material), however, detects protocadherin-15–CD3 at the tips of all the stereocilia (shortest, intermediate, and longest) in outer hair cells from P10 onward, although not in inner hair cells (Fig. 4f,g). As yet, it is unclear why there are these differences in reactivity to various CD3 antisera, but they could be attributable to cell- and time-dependent variations in posttranslational modifications or epitope accessibility.

The distributions of protocadherin-15–CD1 and protocadherin-15–CD3 in vestibular hair cells were also examined with immunogold electron microscopy using anti-peptide antibodies PB303 and PB375 (Fig. 5). Consistent with the immunofluorescence staining, postembedding immunogold labeling reveals that protocadherin-15–CD1 is essentially excluded from the tip region of hair-cell stereocilia (Fig. 5a,b). Very few gold particles are observed in sections stained with non-immune rabbit IgG (Fig. 5c). Both postembedding (Fig. 5d) and pre-embedding (Fig. 5e,f) immunogold labeling reveal that protocadherin-15–CD3 is distributed in a fairly uniform manner around the distal tips of the shorter stereocilia.

Two antibodies raised to sequences in the ectodomain of protocadherin-15 (validated as described above) (supplemental Figs. S2, S3, available at www.jneurosci.org as supplemental material) stain developing but not mature cochlear hair bundles (Fig. 6). One of these antibodies, PB473-3, an antibody to a peptide sequence located immediately upstream of the first cadherin repeat of protocadherin-15 (Fig. 1), only stains the stereocilia of developing mouse cochlear hair bundles in the presence of TX-100 (Fig. 6a–c). The other antibody, HL5614, raised to a recombinant fusion protein encompassing the first two cadherin repeats of protocadherin-15 (Fig. 1), only stains the stereocilia of developing mouse cochlear hair bundles if they are treated with the calcium chelator BAPTA before fixation (Fig. 6d–f). In the absence of BAPTA treatment before fixation, staining of the kinocilium is readily detectable. A pronounced apical-to-basal gradient of staining is observed in the early postnatal mouse cochlea with both ectodomain antibodies, with hair bundles in the apical coil (Fig. 6b,e) staining more intensely than those in the basal coil (Fig. 6c,f), as observed with antibodies specific for protocadherin-15–CD2 (Fig. 2d–f).

The avian tip-link antigen is protocadherin-15

The TLA mAb G19, a species-specific antibody that recognizes an extracellular epitope specifically associated with the tip and kine-
cilial links of chick hair cells and the calyceal processes of photoreceptors, immunoprecipitates two proteins of 200 and 250 kDa from chicken retina (Goodyear and Richardson, 2003). These relative mobilities are similar to those predicted for the longer isoforms of protocadherin-15. To identify these TLA immunoreactive proteins, they were immunopurified from chicken retina, resolved by one-dimensional gel electrophoresis, and analyzed by mass spectrometry (LC-MS/MS). Two peptide sequences from each of the bands (TLA1 and TLA2) match sequences corresponding to two evolutionarily conserved regions of the extracellular domain of human, mouse, and chicken protocadherin-15 [YLTLLQPVDR (GenBank accession numbers NP_149045, AAG53891; MASCOT score of 75) and FQVIATDDYGK (GenBank accession numbers DQ354419; MASCOT score of 85)]. Using the same LC-MS/MS data files, a search of the chicken proteome identifies 8 and 12 peptides, respectively, from the 250 and 200 kDa TLA proteins (Table 1; Fig. 7b, red rectangles). All of these are statistically significant matches to chicken protocadherin-15 and are unique peptides in the chicken proteome. One of these peptides was located in the CD1 domain of chicken protocadherin-15.

Data from Western blot analyses confirm these findings, revealing that the two bands immunoprecipitated by mAb G19 from the avian retina (Fig. 7a, bands 1, 2) are recognized by the antibody directed against the peptide in the extracellular domain of mouse protocadherin-15 (Fig. 7a, PB473-3). Mouse protocadherin-15 and the TLA are thus indistinguishable by Western blot analyses. The TLA1 band with the slower mobility is also recognized by the antibodies directed against the cytoplasmic domain of mouse protocadherin-15–CD1 (Fig. 7a, PB303) and by a polyclonal mouse antiserum directed against a recombinant fragment of chicken CD1 (Fig. 7a, M110), as expected from the mass spectrometric analysis (Table 1). Antibodies directed against mouse protocadherin-15-CD3 detect a minor band that is also immunoprecipitated by the TLA mAb and migrates just slightly faster than the upper TLA1 band (Fig. 7a, HL5383). The TLA2 band is not recognized by antibodies to mouse or chick CD1 (Fig. 7a, PB303, M110) or an antibody to mouse protocadherin-15-CD3.

**Table 1. Mass spectrometric sequencing of chicken TLA**

<table>
<thead>
<tr>
<th>Number</th>
<th>Peptide sequence</th>
<th>Protocadherin-15 location</th>
<th>TLA1</th>
<th>TLA2</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>TGPPATIVPIDEESR</td>
<td>N terminus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>GTAAGPDPIEESLR</td>
<td>EC1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>DNVDYVWLDPSQDR</td>
<td>EC1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>YYVIVQANDR</td>
<td>EC2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>YLTLLQPVDR</td>
<td>EC4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>TGAIIINQADFR</td>
<td>EC6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>TQEALEYFALNK</td>
<td>EC8</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>YTGEILSLK</td>
<td>EC8</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>DNNDYSPVSOK</td>
<td>EC8</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>VNLNEEPSTVK</td>
<td>EC11</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>LVVIAYDDSPYK</td>
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<td>+</td>
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<td>between EC11 and TM</td>
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<tr>
<td>15</td>
<td>VDISSPLFQK</td>
<td>CD1</td>
<td>-</td>
<td>+</td>
</tr>
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Peptide sequences identified in the chicken genome from tryptic digests of the TLA1 and TLA2 bands. The peptides highlighted in bold are conserved in human and mouse protocadherin-15. TM, Transmembrane domain.
time constant of Tip links reappear after the removal of BAPTA with a recovery subtilisin-resistant structures (Goodyear and Richardson, 2003).

et al., 2005). Kinocilial links are, like tip links, BAPTA-sensitive/BAPTA or subtilisin (Goodyear and Richardson, 1999; Goodyear structures, and ankle links are abolished by treatment with either agent, shaft connectors are unaffected by treat-

ment with either BAPTA or subtilisin, but both agents cause a loss of protocadherin-15–CD3 immunoreactivity from the stereocilia (Fig. 9g–i). The ectodomain epitopes detected by the anti-peptide antibody PB473-3 in the presence of TX-100 are unaffected by either calcium chelation or subtilisin treatment (data not shown), whereas those detected by antibody HL5614 to the recombinant fusion protein that require calcium chelation for visualization are subtilisin sensitive (Fig. 9j–l).

The ectodomain epitopes that are revealed by calcium chelation rapidly disappear when calcium is replaced, within 5 min at room temperature after a 5 min exposure to BAPTA (Fig. 10a,b). This is most likely attributable to rapid remasking rather than shedding or loss of the ectodomain because a subsequent, second brief BAPTA treatment reveals the epitopes recognized by this antibody again (Fig. 10c). Protocadherin-15–CD3 reappears more slowly after BAPTA treatment. None is detectable after a 1 h recovery period at 37°C, and complete recovery is observed within 4–24 h (Fig. 10d–f).

Discussion

The results reveal that multiple protocadherin-15 transcripts are expressed in the mouse inner ear and define four major isofrom classes, two of which have entirely novel and previously unidentified cytoplasmic domains. Antibodies specific for the three alternative cytoplasmic domains reveal that three of the four iso-
Protocadherin-15–CD1 and protocadherin-15–CD3 are both expressed in the mature hair bundle, but neither is distributed in a manner that indicates they are uniquely associated with one particular link type found in mature hair bundles. These isoforms are, however, found where the basal end of the tip link attaches to the tip of the shorter stereocilium (CD3) and where the distal end attaches to the side of the adjacent taller stereocilium (CD1), so both isoforms could potentially be associated with the tip-link complex (supplemental Fig. S5, available at www.jneurosci.org as supplemental material). The results of this study further show that the TLA, a previously unidentified antigen known to be specifically associated with the tip and kinocilial links of sensory hair cells, is a mixture of two isoforms, one reactive with antibodies to mouse protocadherin-15–CD1 and the other reactive with antibodies to mouse protocadherin-15–CD3.

The previously reported protocadherin-15–CD1 is not found on the surface of immature cochlear hair bundles at the apex of the early postnatal (P3) mouse cochlea and is therefore unlikely to play a role in the early stages of hair-bundle development. In mature hair cells, protocadherin-15–CD1 is not found on the kinocilium (when present) and, although concentrated toward the tops of the stereocilia in vestibular hair cells, is clearly excluded from the extreme tip region of each stereocilium in both auditory and vestibular hair cells. In contrast to a recent study (Senften et al., 2006), protocadherin-15–CD1 was never found to be concentrated in the ankle-link region of the hair bundle, even during the postnatal stages of development (P2–P9) when ankle links are prominent (Goodyear et al., 2005).

An isoform from another novel class, protocadherin-15–CD3, is concentrated at the tips of developing stereocilia and expressed along the length of their shafts but becomes restricted to the tips of stereocilia as the hair bundle matures. The mechanisms that direct the various protocadherin-15 isoforms to different domains within the hair bundle remain to be elucidated but may involve molecules that interact with the unique PDZ ligands or other motifs exclusive to each of the cytoplasmic domains. Harmonin, a scaffold protein that has many alternative splice isoforms, and myosin VIIA have been shown to interact in vitro with the CD1 domain of protocadherin-15 (Adato et al., 2005; Reiners et al., 2005; Senften et al., 2006) and may be involved in the localization of protocadherin-15 in stereocilia.

Protocadherin-15–CD1 and protocadherin-15–CD3 form classes have very different spatiotemporal expression patterns in developing and mature hair cells (supplemental Fig. S5, available at www.jneurosci.org as supplemental material), indicating a specific role for each protocadherin-15 isoform class in the ontogeny, structure, and function of the sensory hair bundle.

Isoforms from one of the novel classes, protocadherin-15–CD2, are expressed along the lengths of stereocilia during the early stages of hair-bundle development, but become progressively localized toward the tip of the bundle in more mature hair cells and are then lost from the stereocilia. Isoforms from this class are not expressed by mature cochlear hair cells and are only present in the kinocilium of mature vestibular hair cells. The gradient of staining that diminishes toward the basal turn of the cochlea in the early postnatal cochlea supports the possible involvement of protocadherin-15–CD2 in the formation of the transient lateral links that are found in abundance on developing cochlear hair bundles (Goodyear et al., 2005). This expression pattern is similar to that described previously for cadherin-23 (Lagziel et al., 2005; Michel et al., 2005) and suggests that cadherin-23 and protocadherin-15–CD2 may contribute collectively to the transient lateral links and also to the kinociliary links of mature hair cells.

Figure 8. Colocalization of the TLA and protocadherin-15. a–c, Confocal images of the chicken utricular macula double labeled for the PB473-3 peptide (a, red) and the TLA using mAb G19 (b, green). Image in c is a merge of images shown in a and b. Scale bar, 10 μm. d–f, Double-immunogold labeling for the PB473-3 peptide (5 nm particles; arrowheads) and the TLA (10 nm particles; arrows) in the tip-link region (d, e) and in the stereocilium–kinocilium link region (f) of utricular macular hair cells. S, Stereocilium; K, kinocilium. Scale bars, 100 nm.
An antibody raised to the peptide sequence potentially common to the ectodomain of all mouse protocadherin-15 isoform classes stains chick hair bundles in a manner similar to that observed with the anti-TLA mAb, further confirming the TLA is protocadherin-15. Although this antibody and a second antiserum raised to a recombinant fragment encompassing the same peptide and the first two cadherin repeats (also expected to react with isoforms from all four classes of protocadherin-15) both reacted intensely with embryonic mouse hair bundles, they failed to stain mature mouse hair bundles. In the developing mouse cochlea, reactivity to these antibodies diminished rapidly during the early postnatal stages of development, and the spatiotemporal staining pattern observed with these antibodies is similar to that observed with the antibody raised to the cytoplasmic domain of protocadherin-15–CD2. The protocadherin-15–CD1 and protocadherin-15–CD3 isoforms expressed in the mouse may lack the regions of sequence recognized by these ectodomain antibodies, or these regions may somehow be masked and inaccessible in the mouse. In this respect, it should be noted that TX-100 was, for reasons that are as yet unclear, required to visualize protocadherin-15 with the antibody to the extracellular peptide sequence and that BAPTA treatment was necessary to detect protocadherin-15 with the antibody to the recombinant ectodomain fragment. In the mouse, BAPTA and TX-100 appear to only unmask the epitopes associated with the ectodomain of protocadherin-15–CD2 and not those associated with the ectodomains of protocadherin-15–CD1 or protocadherin-15–CD3.

The data discussed above indicate that protocadherin-15–CD1 and protocadherin-15–CD3 may both be associated with the tip-link complex, and several other lines of evidence indicate that protocadherin-15 is required for mechanotransduction. First, receptor potentials cannot be detected from the lateral line...
organisms of zebrfish orbiter mutants with mutations in the extra-cellular domain of Pcdh15 despite normal-appearing hair bundles (Nicolson et al., 1998; Seiler et al., 2005). Second, vestibular evoked potentials cannot be detected in av mice with mutations in Pcdh15, and the mature utricular hair cells of the av-2f and av-3f mutant do not load with AM1-43 (Alagramam et al., 2005), a fixable version of FM1-43 [N-(3-triethylammoniumpropyl)]-4-(4-(dibutylamino)styryl) pyridinium dibromide], a styryl dye that is known to enter and accumulate in hair cells via their transducer channels (Gale et al., 2001; Meyers et al., 2003). Third, transduction currents cannot be recorded using whole-cell patch clamping in either the vestibular or cochlear hair cells of early postnatal av-3f mice, and neither cell type loads with the fixable form of FM1-43, FM1-43FX (Senften et al., 2006). Fourth, one of the two isoform classes that is potentially associated with tip links, protocadherin-15–CD3, is rapidly lost from the hair bundle in response to calcium chelation, a condition that is known to abolish transduction (Assad et al., 1991). Protocadherin-15–CD3 also reappears over the period transduction (Zhao et al., 1996), and FM1-43 dye loading (Gale et al., 2001) recovers after the removal of calcium chelators. Although the results further indicate the ectodomain of protocadherin-15 can rapidly refold on the cell surface after removal of BAPTA, this is a very rapid process and one that foreshadows the recovery of transduction.

Despite this evidence, it remains debatable whether protocadherin-15 is a component of the central strand of the tip link, a component of the “anchoring” elements of the tip link, peripherally associated with the tip-link complex, and/or required for tip-link formation. Tip links can be up to 250 nm in length and are composed of two helical filaments along most of their length (Kachar et al., 2000; Tsuprun et al., 2004). The predicted extracellular domain of protocadherin-15 has a juxtamembrane region with a mass of 53 kDa and unknown tertiary structure and a distal region with a mass of 11 EC repeats, each of which spans 4.3 nm (Boggon et al., 2002). Even as a trans dimer, protocadherin-15 is unlikely to be long enough to form a tip link. There is evidence that the tip link splay at both ends into two or more strands and that it is attached to the tip of the shorter stereocilium by three anchoring filaments (Kachar et al., 2000). In the mouse, protocadherin-15–CD3 could therefore form the anchoring filaments seen at the basal end of the tip-link complex, and protocadherin-15–CD1 could contribute to the additional strands seen its distal end. Cadherin-23 has been proposed to be a candidate for the tip-link filament (Siemens et al., 2004), and it is possible that cadherin-23 and protocadherin-15 both contribute to the structure of the tip-link complex. Although the ectodomain of cadherin-23 recognized by antibody N1 (Michel et al., 2005) and that of protocadherin-15 recognized by antibody HL5614 are, unlike tip links and transduction (Goodyear and Richardson, 1999; Michel et al., 2005), both subtilisin sensitive, their association with other proteins or as yet unidentified posttranslational modifications may alter the protease sensitivity of molecules that are an integral part of the tip-link complex.

In summary, the results of this study demonstrate the TLA, a protein associated with the tip-link complex of sensory hair cells, is protocadherin-15. Additional studies will now be required to determine whether protocadherin-15 is an integral or peripheral component of this structure and whether it interacts either directly or indirectly with any of the other components of the tip-link complex.

References


Lagziel A, Ahmed ZM, Schultz JM, Morell RJ, Belyantseva IA, Friedman TB (2005) Spatiotemporal pattern and isoforms of cadherin 23 in wild type