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Origin of the tetraspanin uroplakins and their co-evolution with associated proteins: Implications for uroplakin structure and function

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Abstract

Genome level information coupled with phylogenetic analysis of specific genes and gene families allow for a better understanding of the structure and function of their protein products. In this study, we examine the mammalian uroplakins (UPs) Ia and Ib, members of the tetraspanin superfamily, that interact with uroplakins UPII and UPIIIa/IIIb, respectively, using a phylogenetic approach of these genes from whole genome sequences. These proteins interact to form urothelial plaques that play a central role in the permeability barrier function of the apical urothelial surface of the urinary bladder. Since these plaques are found exclusively in mammalian urothelium, it is enigmatic that UP-like genomic sequences were recently found in lower vertebrates without a typical urothelium. We have cloned full-length UP-related cDNAs from frog (*Xenopus laevis*), chicken (*Gallus gallus*), and zebrafish (*Danio rerio*), and combined these data with sequence information from their orthologs in all the available fully sequenced and annotated animal genomes. Phylogenetic analyses of all the available uroplakin sequences, and an understanding of their distribution in several animal taxa, suggest that: (i) the UPIa/UPIb and UPII/UPIII genes evolved by gene duplication in the common ancestor of vertebrates; (ii) uroplakins can be lost in different combinations in vertebrate lineages; and (iii) there is a strong co-evolutionary relationship between UPIa and UPIb and their partners UPII and UPIIIa/IIIb, respectively. The co-evolution of the tetraspanin UPs and their associated proteins may fine-tune the structure and function of uroplakin complexes enabling them to perform diverse species- and tissue-specific functions. The structure and function of uroplakins, which are also expressed in *Xenopus* kidney, oocytes and fat body, are much more versatile than hitherto appreciated.
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1. Introduction

Uroplakins are the integral membrane protein subunits of urothelial plaques (also known as asymmetric unit membrane or AUM; Sun et al., 1999; Wu et al., 1994, 1990) that line the highly specialized apical surface of the mammalian urinary bladder epithelium (Apodaca, 2004; Hicks, 1965; Lewis, 2000; Porter and Bonneville, 1963; Sun et al., 1996; Vergara et al., 1969). There are four major uroplakins (UPs) Ia, Ib, II and IIIa (Lin et al., 1994; Walz et al., 1995; Wu et al., 1990; Wu and Sun, 1993; Yu et al., 1994), and a minor UPIIIb (Deng et al., 2002). Chemical crosslinking and co-transfection studies indicated that

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UPIa and UPIb interact with UPII and UPIIIa (or UPIIIb), respectively, forming two heterodimers that can then exit the endoplasmic reticulum (Deng et al., 2002; Hu et al., 2005; Tu et al., 2002; Wu et al., 1995). Further interactions between the heterodimers lead to the formation of the 16-nm particle that are packed hexagonally forming urothelial plaques (discussed in Hu et al., 2005). Genetic ablation of uroplakins results in a lack of plaques and a leaky urothelium indicating that uroplakins are the integral protein subunits of the urothelial plaques which contribute to the remarkable permeability barrier function of mammalian bladder urothelium (Hu et al., 2000, 2002; Kong et al., 2004).

The mammalian UPIa and UPIb proteins, both ~260 amino acids in length, are ~39% similar and belong to the tetraspanin superfamily that contains many cell surface proteins playing important roles in immunological signaling, growth regulation, cell motility, viral infections and membrane architecture (Berditchevski, 2001; Boucheix and Rubinstein, 2001; Hemler, 2003; Kovalenko et al., 2005; Levy and Shoham, 2005a,b; Maecker et al., 1997; Martin et al., 2005; Tarrant et al., 2003; Yunta and Lazo, 2003). As the name 'tetraspanin' implies, all members of this family traverse the lipid bilayer four times, with three of the transmembrane domains clustered near the N-terminus and one, separated from the third by a large (~150 amino acids) extracellular loop, close to the C-terminus (Yu et al., 1994).

Uroplakins II and IIIa (the partner proteins of UPIa and Ib) are about 184 and 287 amino acids in length, respectively (Lin et al., 1994; Wu and Sun, 1993). In addition, UPII and UPIIIa share a juxta-membrane stretch of about 12 amino acids on the extracellular side of the single transmembrane domain (Lin et al., 1994; Wu and Sun, 1993). Since uroplakins Ia/II and Ib/IIIa form nearly stoichiometric complexes and can be isolated in large quantities, uroplakins provide an excellent model for studying the structure, function and molecular evolution of tetraspanins and their partner proteins.

Since urothelial plaques are highly characteristic of the apical surfaces of mammalian urothelium, it is enigmatic that uroplakin-related genomic sequences are found in frog (Mahbub Hasan et al., 2005; Sakakibara et al., 2005), and in fish and chicken based on available genebank sequences (see below). In this study, we confirm the uroplakin identities of such non-mammalian genes by cDNA cloning and sequencing. Analysis of these genes in frog, zebrafish, and chicken suggest that these genes may be functional in a manner distinct from the mammalian uroplakins. In order to understand the genomic origin of these genes in a wide array of animal taxa, we have also conducted a phylogenetic analysis of all the available orthologs from all fully sequenced and annotated genomes in the database. In addition, we have examined the evolutionary history of these proteins in order to discover patterns of change that might shed light on the structure and function of the tetraspanin uroplakins and their associated proteins.

2. Materials and methods

2.1. Database mining and sequence analyses

All sequences used in this study are listed in Supplementary Table 1. We first searched all of the complete genome sequences and partially completed genomes for uroplakins Ia, Ib, II, IIIa and IIIb using the BLAST functions on the genome tools websites for each of the sequenced genomes with the human forms of these proteins as query sequences. For our primary sequence analyses we used only uroplakin sequences from fully sequenced and reasonably well-annotated genomes *Mus musculus* (Mm, mouse), *Rattus norvegicus* (Rn, rat), *Homo sapiens* (Hs, human), *Danio rerio* (Dr, zebrafish), *Gallus gallus* (Gg, chicken), and *Ciona intestinallis* (Ci, sea squirt). In addition, we included uroplakins from well-characterized EST of *Pan troglodytes* (Pt, chimpanzee), *Sus scrofa* (Ss, pig), *Canis familiaris* (Cf) and *Bos taurus* (Bt), *Xenopus tropicalis* (Xt), *Xenopus laevis* (Xl), *Ambystoma mexicanum* (Am, axolotl), *Oncorhynchus mykiss* (Om, rainbow trout), *Ictalurus punctatus* (Ip, catfish), *Cyprinus carpio* (Cc, carp), *Squalus acanthias* (Sa, spiny dogfish), and *Leucoraja erinacea* (Le, little skate). Analysis of the co-evolution of UPIa with UPII and UPIb with UPIII included sequences of these genes from several mammals where all four UPs are available in the database (see Fig. 5 for a list of these species).

2.2. Cloning and sequencing of cDNAs

Total RNAs were purified from *Xenopus laevis*, *Danio rerio* and *Gallus gallus* using a TRIZOL kit (Invitrogen, Madison, WI), and used to synthesize cDNA using a SuperScript kit (Invitrogen) with oligo(dT) primer. The cDNAs of various uroplakin-orthologues were isolated by RT-PCR using primers based on the hypothetical sequences assembled from the available cDNA/EST data using SEQMAN (DNASTAR, Carlsbad, CA). The primer sequences used for full-length ORF amplification in *Xenopus* were Ia (sense: 5′-gggagctgccagacaagttgggctc-3′ and antisense: 5′-ggggataatgtggctcctcagttcat-3′); Ib (5′-aggacaggtgtttcccatctctc-3′ and 5′-gctggccaagatagtgtagacct-3′); II (5′-acccacgcgtccgagaggcatc-3′ and 5′-gttagatgaacatcaaaaggacgc-3′); IIIa (5′-tgctgatgtgagagtgtacctgacac-3′ and 5′-tagacgtttccataggtggaaatg-3′); and IIIb (5′-ttgctttctaagcactgccatacgc-3′ and 5′-catactgtttatagtattttgacagata-3′). The primers sequence of chicken full-length uroplakin Ib used were 5′-ttctgcatcaccagcagggaa-3′ and 5′-aatggagcaggagacgagtgaatgc-3′. The cDNA products were cloned into a pGEM-T vector (Promega, Madison, WI) and sequenced.

2.3. Northern blot analyses

Five micrograms of total RNA was fractionated on a formaldehyde agarose gel, and transferred onto a nylon membrane (Hybond-XL; Amersham Corp., UK). The blot was hybridized with ^{32}P-labeled cDNA at 45 °C overnight

Ia

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D MG-------A-VTCLMVT-VVGLNAIAAAAGLALSAVAIWVAVDGYKLYPISGVSGKDDIFAGAWIAIFTGFAFFLTCIFGIFAALKRSRALMLVYLIIM
X MA-------EKGSSGMVTFIVFGNIVILLSGLALFAETIWATTDPYKVYPILGVTGKDDVFAGGWIAIFCGFSFFILGVFGILAVQRGSRTMVLTYLVLM
M MASAAT-EGEKGSPVVVGLLVVGNIIILLSGLALFAETVWVTADQYRVYPLMGVSGKDDVFAGAWIAIFCGFSFFVVASFGVGAALCRRRYMILTYLLLM
B MASAAAATTEKGSPVVVGLLVMGNIIILLSGLALFAETVWVTADQYRIYPLMGVSGKDDVFAGAWIAIFCGFSFFVVASFGVGAALCRRRSMILTYLILM
H MASAAAAEAEKGSPVVVGLLVVGNIIILLSGLSLFAETIWVTADQYRVYPLMGVSGKDDVFAGAWIAIFCGFSFFMVASFGVGAALCRRRSMVLTYLVLM

D FIIFLFESASAITSATNRDYLVGNSNLVKKQMLQYY-ADSST-QGQQITMTWNNVMTQVQCCGADGPTDWIQYNSTYRQLFGAAS-LWPLGCCKRQSSNF
X MIVYIFECASCITSFTHRDYM-INSNVIKGQMLTYYS-DSSTPQGRDVTGVWLRMMLEKNCCGVDGPLDWVDYSSTFRKTYNETTAPWPLWCCQRDS-NF
M LIVYIFECASCITSYTHRDYMVSNPSLITKQMLTYYSAD--TDQGQELTRLWDRIMIEQECCGTSGPMDWVNYTSAFRAATPEVVFPWPPLCCRRTG-NF
B LIIYIFECASCITSYTHRDYMVSNPSLITKQMLTFYSADS--NQGRELTRLWDRIMIEQECCGTSGPMDWVNFTSAFRATTPEVVFPWPPLCCRRTG-NF
H LIVYIFECASCITSYTHRDYMVSNPSLITKQMLTFYSAD--TDQGQELTRLWDRVMIEQECCGTSGPMDWVNFTSAFRAATPEVVFPWPPLCCRRTG-NF

D EVVDPIGCKAGVTSSMFTQGCFQYIESVLSRYTWAVSWYGFSVLMLVFFTLVIAMIY-YTQ-LP250
X QIINQQGCVVGLKSYVYQQGCFDHISNAINSYTWGISWFGFAILMWTMIVMLVTM-YNYTKMN252
M IPINEDGCRVGHMDYLFTKGCFEHIGHAIDSYTWGISWFGFAILMWTLPVMLIAM-YFYTT-L257
B IPVNEEGCRLGHLDYLFTKGCFEHIGHAIDSYTWGISWFGFAILMWTLPVMLIAM-YFYTT-L258
H IPLNEEGCRLGHMDYLFTKGCFEHIGHAIDSYTWGISWFGFAILMWTLPVMLIAM-YFYT-ML258
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Ib

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X M-KDDSGIRCFQSLLIFGNVVIGLCGLALTAECIFFVSDQSGIYPLLEATDNDDIFGAAWIGIFAGFCLFVLSILGIIGIMKSNRRMLMVYLILMFIVYA
M MAKDDSTVRCFQGLLIFGHVIVGMCGIALTAECIFFVSDQHSLYPLLEATNNDDIFGAAWIGMFVGICLFCLSVLAIVGIMKSNRKILLAYFIMMFIVYG
B MAKDDSTVRCFQGLLIFGNVIIGMCSIALMAECIFFVSDQNSLYPLLEATNNDDIYAAAWIGMSVGICLFCLSVLGIVGIMKSNRKILLVYFILMFIVYA
H MAKDNSTVRCFQGLLIFGNVIIGCCGIALTAECIFFVSDQHSLYPLLEATDNDDIYGAAWIGIFVGICLFCLSVLGIVGIMKSSRKILLAYFILMFIVYA

X FEVASAITAATQQNFFIPELFLKQMLEFYQNPNPINNDNLWKINGVTRTWNRFMLLNGCCGVNGPQDWQTYNSVFRQFNSDSAYPWPQQCCVMNSLGQPV
M FEVASCITAATQRDFFTTNLFLKQMLMRYQNNSPPTNDDEWKNNGVTKTWDRLMLQDHCCGVNGPSDWQKYTSAFRVENNDADYPWPRQCCVMDKLKEPL
B FEVASCITAATQRDFFTPNLFLKQMLERYQNNSPPNNDDQWKNNGVTKTWDRLMLQDNCCGVNGPSDWQKYTSAFRTENSDADYPWPRQCCVMNSLKEPL
H FEVASCITAATQRDFFTPNLFLKQMLERYQNNSPPNNDDQWKNNGVTKTWDRLMLQDNCCGVNGPSDWQKYTSAFRTENNDADYPWPRQCCVMNNLKEPL

X NLDACKLGVAGYVNLNGCYDLMAGPMTRHAWGVAWFGFSILCWTFWVLLGSMFYWTRIEY259
M NLDACKLGVPGYYHSQGCYELISGPMDRHAWGVAWFGFAILCWTFWVLLGTMLYWSRIEY260
B NLDACKLGVPGYYHSHGCYELISGPMNRHAWGVAWFGFAILCWTFWVLLGTMFYWSRIDY260
H NLEACKLGVPGFYHNQGCYELISGPMNRHAWGVAWFGFAILCWTFWVLLGTMFYWSRIEY260
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II

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X MQ-------LLWITAVL-LLISGAIAQ-NTSLADGVLTP-LSTSVIIAFPGCKDSGKTVNLIVANGTTT-VQNISLQVPQCRLKRDVVVINNSQSGNVQT
M MASTLPVQTLPLILILLAVLAPG-TADFNISSLSGLLSPALTESLLIALPPCHLTGGNATLMVRRANDSKVVKSDFVVPPCRGRRELVSVVDSGSGYTVT
B MASPWPVWTLSWILILLAVLVPGAAADFNISSLSGLLSPVMTESLLVALPPCHLTGGNATLTVRRANDSKVVRSSFVVPPCRGRRELVSVVDSGSGFTVT
H MAPLLPIRTLPLILILLALLSPG-AADFNISSLSGLLSPALTESLLVALPPCHLTGGNATLMVRRANDSKVVTSSFVVPPCRGRRELVSVVDSGAGFTVT

X VNVGYQIQNLQPGAIYTTYYAVD-------GSNIPSITFSTRSVSQTVPDIMARSGGMVVITVLLSIAMFVLLVGLIAVLVIG-RK167
M RLSAYQVTNLTPGTKYYISYRVQKGTSTESSPETPMSTLPRKNM-ESIGLGMARTGGMVVITVLLSVAMFLLVVGLIVALALGARK184
B RLSAYQVTNLAPGTKYYISYLVTKGASTESSREIPMSTFPRRKA-ESIGLAMARTGGMVVITVLLSVAMFLLVLGLIIALALGARK185
H RLSAYQVTNLVPGTKFYISYLVKKGTATESSREIPMSTLPRRNM-ESIGLGMARTGGMVVITVLLSVAMFLLVLGFIIALALGSRK184
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IIIa

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X MGPWRYLFGL-CWFLQVHFARSAVPLLANSDFFSLNPTQTTITLERPFCMF--KDAID----VYLFAIVKGAT--NIQVADAAKKVIASNYTGTQGGLLG
M MLLLWALLALGC--LRCGWTVNLQPQLASVTFATNNPTLTTVALEKPLCMFDSSEPLSGSYEVYLYAMVDSAMSRNVSVQDSAGVPLSTTFRQTQGGRSG
B MPPLWVVLALGC--LRLGSGVNLQPQLASVTFATNNPTLTTVALEKPLCMFDSSAALHGTYEVYLYVLVDSASFRNASVQDSTKTPLSSTFQQTQGGRTG
H MPPLWALLALGC--LRFGSAVNLQPQLASVTFATNNPTLTTVALEKPLCMFDSKEALTGTHEVYLYVLVDSAISRNASVQDSTNTPLGSTFLQTEGGRTG

X PYQVAKLDNPKCENIQASNIMADP-------NKYIVRVGGDVNCLTDPNFKGICNPPLQNNLQYRFTYVFT--IGDVVQYQTDWSPPISTVNVKSSGTID
M PYKAAAFDLTPCGDLPSLDAVGDVTQASEILNAYLVRVGNNGTCFWDPNFQGLCNPPLTAATEYRFKYVLVNMSTGLVQDQTLWSDPIWTNRPIPYSAID
B PYKAAAFDLTPCSDSPSLDAVRDVSRASEILNAYLIRVGTNGTCLLDPNFQGLCNPPLSAATEYRFKYVLVNMSSGLVQDQTLWSDPIRTDRLTLYSAID
H PYKAVAFDLIPCSDLPSLDAIGDVSKASQILNAYLVRVGANGTCLWDPNFQGLCNAPLSAATEYRFKYVLVNMSTGLVEDQTLWSDPIRTNQLTPYSTID

X TWPGRRSGGMIVLTSILSTLMFF--VFFAY-IVGFAYSIL-NGSQTKEVSRHDTQTT--AVLQK---AQEPGDITYSSTLAG-----SERYAATQQA265
M TWPGRRSGGMIVITSILGSLPFFLLVGFAGAIIL---SFVDMGSSDGEMT-HDSQITQEAVP-KTLGTSEP---SYSSVNRGPPLDRAEVFSSKLQD287
B TWPGRRSGGMIVITSILGSLPFFLLIGFAGAIVL---SLVDRGDADGATS-HDSQITQEAVP-KSLGTSEP---SYTSVNRGPSLDRAEVYASKLQD287
H TWPGRRSGGMIVITSILGSLPFFLLVGFAGAIAL---SLVDMGSSDGETT-HDSQITQEAVP-KSLGASES---SYTSVNRGPPLDRAEVYSSKLQD287
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IIIb

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X MDFHIK--------VILAIATCALSVGADITTYVPQLTLMPIQGSVTSTTFTLDKPQCIF-G-SRT-NQVWLLVARSNVSVSITNAM-LTPPSMYSSFPT
M MVRTRWQPHPPPPLLLLVLVWLPQSLSLDLIAYVPQITAWDLEGKITATTFSLEQPRCVFDEHVSTKDTIWLVVAFSNASRDFQNPQTAAKIPTFPQLLT
B MGLPSRQPRLWL-LLLVVLGWPQPCLTLDLIPYTPRITSWDLEGKVTATTFSLEQPRCVLDRHSSAADTVWLVVAFSNASRVFQNPQTLAEIPASPRLLT
H MGLPWGQPHLGLQMLLLALNCLRPSLSLELVPYTPQITAWDLEGKVTATTFSLEQPRCVFDGLASASDTVWLVVAFSNASRGFQNPETLADIPASPQLLT

X QGYY-HVPLGTEASYPCSNTAD------YIRVGDTVYC----TDNTYCNARLPDSGPYRVKFVVMNNNAL-VSSSLWSGLITLRTGKNPSTIDTWPGRRS
M DGHYMTLPLSLDQ-LPCEDLTGGSGGVPVLRVGNDFGC----YQRPYCNAPLPSQGPYSVKFLVMDAAGPPKAETKWSNPIYLHQGKNPNSIDTWPGRRS
B DGHYMTLPLTMDQ-LPCEDPADGSGRAPVLRVGNDAGCLADLHQPRYCNAPLPGPGPYRVKFLLTNSRGSPQAETRWSDLIALRQGKSPGSIDTWPGRRS
H DGHYMTLPLSPDQ-LPCGDPMAGSGGAPVLRVGHDHGC----HQQPFCNAPLPGPGPYRVKFLLMDTRGSPRAETKWSDPITLHQGKTPGSIDTWPGRRS

X GGMIVLTSILSLLMGILTLCLIAAFFVGCKGMSRKKGTKEKSIIQADQNTKNYKTHY--SS---TIR--HQP--DP-PS-SPEPKIV252
M GCMIVITSILSALAGLLLLAFLAASTTRFSSLWWPEEAPEQLRIGSF-MGKRYMTHHIPPSEAATLPVGCEPGLDPLPSLSP276
B GDMIIITSILSSLAGLLLLAFLAASSVRFSSLWWPEEAPEQLRIGSF-MGKRYMTHHIPPSEAATLPVGCEPGLERFPSLSP279
H GSMIVITSILSSLAGLLLLAFLAASTMRFSSLWWPEEAPEQLRIGSF-MGKRYMTHHIPPSEAATLPVGCKPGLDPLPSLSP276
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Fig. 1. Pileup alignment of the amino acid sequences of uroplakins of zebrafish (D), *Xenopus* (X), bovine (B), human (H) and mouse (M).

in hybridization buffer (ULTREhyb; Ambion Inc., Austin, TX). After discarding the hybridization buffer, the blot was washed at 45 °C for 2 × 5 min in 2× SSC, 0.1% SDS and 2 × 15 min in 0.1× SSC, 0.1% SDS. After autoradiography, the probes were stripped off by washing the blot in sterile H_2O containing 0.5% SDS at 95 °C for 10 min followed by rehybridizing with other uroplakins and EF1-α probes.

2.4. Phylogenetic analyses

Alignments of sequences were obtained using ClustalX (Thompson et al., 1997) with default settings in place. All phylogenetic analyses were performed using PAUP* (Swofford, 2001). Parsimony searches were performed using the parsimony ratchet PAUPRAT (Sikes and Lewis, 2001) with 10,000 ratchet replicates and a search on all shortest trees from the ratchet by a heuristic method using the ratchet trees as starting trees with TBR branch swapping and the retention of all shortest trees. Bootstrap and Jackknife trees were also generated using PAUP* (Swofford, 2001) with 1000 resampling replicates. For the uroplakin family analysis, we used one *D. rerio* (TspDr) and two *Ciona* (TspCi and TspCi*) tetraspanin protein sequences as outgroups because they showed close affinity to uroplakins in a larger tetraspanin analysis (A. Garcia-España et al., in preparation). BLAST searches always failed to detect any non UPII, UPIIIa or UPIIIb scores that might indicate close affinity of candidate proteins to use as outgroups (*e* values were always equal to or greater than 0.5). Therefore, there are no clearly definable outgroup candidates for the UPII, UPIIIa and UPIIIb proteins, so we rooted the UPII, UPIIIa and UPIIIb tree by choosing all of the UPII proteins in the data matrix as outgroups and enforcing them as monophyletic in the analysis. We also performed Bayesian analysis on both UP data sets, using the pars model setting in Mr Bayes (Huelsenbeck and Ronquist, 2001). All Bayesian analyses were performed using the MCMC option, 1,000,000 replicates with "burn in" set at 5000 generations.

2.5. Analyses of co-evolutionary relationships

The TreeMap programs (1.01 and 2.3; (Charleston and Page, 2002)) were used to evaluate the co-evolution of the uroplakin genes. Phylogenetic trees were generated for the UPIa and UPIb protein sequences using seven mammalian species whose four UP protein sequences are available in the database (human, chimpanzee, pig, dog, mouse, rat and cow) and two species of frog where all four protein sequences are available in the database. A phylogenetic tree was constructed as well using PAUP (Swofford, 2001) for the UPII and UPIIIa sequences using parsimony. Trees generated from such analysis were input into the TREEMAP program to visualize the co-evolutionary relationships of UPIa with UPII, and UPIb with UPIIIa. The "calculate jungle" option in Treemap 2.3 was used to calculate all potential evolutionary events involved in the co-evolution of these proteins. Jungle solutions give the minimum number of the four evolutionary events involved in co-evolutionary relationships—duplications, lineage extinctions, horizontal transfers and straight co-evolutionary divergence. This approach was originally developed to characterize parasite host interactions. In the current application, the two interacting proteins (UPIa with UPII, and UPIb with UPIIIa) can be thought of as hosts (UPIa and UPIb) and parasites (UPII and UPIIIa) in a co-evolutionary relationship. Once the jungle solutions for co-evolutionary patterns of UPIa with UPII and UPIb with UPIIIa were generated, the significance of such relationships were evaluated using Treemap 2.3. This evaluation involves a test where both the UPI trees and UPII or UPIIIa trees are randomized and then analyzed for the number of the four co-evolutionary events listed above. Results of this analysis are reported as P values at the 95% confidence level when the observed relationships are more economical than 95% of the randomized relationships. In our tests, we randomized both trees simultaneously 1000 times for each test. As controls, we also tested the relationships of non-interacting UP proteins by comparing potential relationships of UPIa with UPIIIa and UPIb with UPII using the same approach outlined above.

3. Results

3.1. Identification of uroplakins of zebrafish, frog and chicken

By searching the completed zebrafish (*D. rerio*) genomic database, we obtained an uroplakin Ia-related, unannotated gene sequence (for the sequences used in this study see Supplementary Table 1). We generated its full-length cDNA sequence by RT-PCR using the total zebrafish RNA as the template (Fig. 1). A similar search of the *X. laevis* and X. *tropicalis* cDNA databases identified frog ESTs related to all known mammalian uroplakins, i.e., UPIa, Ib, II, IIIa and IIIb. We therefore used *X. laevis* bladder cDNAs as the template to isolate by RT-PCR all five full-length uroplakin-related cDNAs, and found that their sequences (Fig. 1) were >95% similar to those of corresponding cDNA sequences in the database. A comparison of the frog and mammalian uroplakins showed that frog UPIa, Ib, II, IIIa and IIIb were 60.9%, 73.6%, 33.5%, 36.3% and 36.1% similar, respectively, to their human counterparts thus confirming their potential uroplakin identity (Table 1; see Section 4). Survey of various frog tissues by Northern blot revealed that frog bladder contained all five uroplakins (Fig. 2, lane 1). Kidney, oocytes and fat body (Fig. 2, lanes 2, 12 and 13)

Table 1
Homologies between the uroplakins of the frog and those of the mammals

	Ia	Ib	II	IIIa	IIIb
Bovine	59.7	73.6	37.4	35.6	37.7
Mouse	61.3	70.9	36.0	36.3	37.4
Human	60.9	73.6	33.5	36.3	36.1
Average	*60.6*	*72.7*	*35.6*	*36.1*	*37.1*

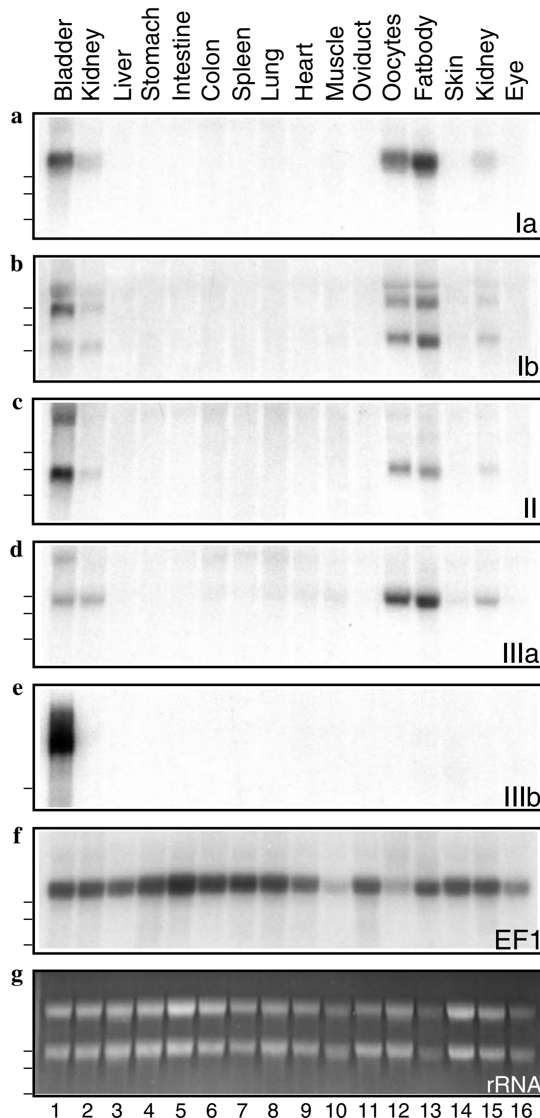

Fig. 2. Expression of uroplakin genes in various frog tissues. Messenger RNAs from various frog tissues were resolved electrophoretically, transferred to a Hybond-XL nylon membrane and probed with partial cDNAs of frog uroplakins Ia (a), Ib (b), II (c), IIIa (d) and IIIb (e). An identical blot was probed using EF1-α cDNA (f) as a control. Bottom panel (g) shows the ethidium bromide-staining of the 28 and 18 s rRNA. Samples are: (1) bladder, (2) kidney, (3) liver, (4) stomach, (5) intestine, (6) colon, (7) spleen, (8) lung, (9) heart, (10) muscle, (11) oviduct, (12) oocytes, (13) fat body, (14) skin, (15) kidney and (16) eye. The bars on the left are RNA size markers, from the top, of 1770-, 1520- and 1280-bases.

contained, however, only uroplakins Ia, Ib, II and IIIa, indicating that UPIIIb was the only "bladder-specific" uroplakin in the frog (Fig. 2e, lane 1). Finally, database search revealed three chicken cDNA/EST sequences that were similar to mammalian uroplakins Ib, IIIa and IIIb; we have confirmed the identify of the UPIb by cloning and sequencing its cDNA (data not shown). We were unable, however, to detect UPIa and UPII cDNAs. An extensive search of the chicken genomic database (Wallis et al., 2004) confirmed the presence of uroplakin Ib, IIIa and IIIb genes, and the absence of the UPIa and UPII genes (Fig. 3).

3.2. Evolutionary relationships among the uroplakins

Phylogenetic analyses of the entire tetraspanin superfamily have shown that the uroplakin Ia and Ib subfamilies were imbedded as a tight clade within the tetraspanin superfamily (Boucheix and Rubinstein, 2001; Hemler, 2003). Fig. 4A shows the gene genealogy obtained when all UPIa and Ib protein sequences from completed and well-annotated genomes, as well as EST sequences, were analyzed using outgroups established from a larger tetraspanin tree (unpublished). The relationships depicted in these trees were strongly supported by several independent robustness analyses (the levels of support for the nodes in the trees, based on bootstrap, jackknife and Bayes statistics, are indicated by different colored circles in Fig. 4; see Section 2). These results also detected, based on a search of the incomplete database, UPIa- and UPIb-related genes in the cartilaginous fishes shark (Ia and Ib) and little skate (Ib), and in the bonny fishes trout (Ib), carp (Ia), catfish (Ia) and zebrafish (Ia; Fig. 4A). The single chicken tetraspanin uroplakin had previously been assigned to the UPIb group, and our analysis supported this assignment (Fig. 4A).

We have also generated a gene genealogy for all of the currently available UPII, UPIIIa and UPIIIb gene sequences (Fig. 4B). Like the genealogy of UPIs, the UPII and UPIII genealogy was consistent with the organismal histories, with the exception of a truncated axolotl UPIII-related sequence that did not cluster with other amphibians (Fig. 4B). EST data mining yielded a UPII-related gene in the little skate, and a UPIII-related gene in the zebrafish and the rainbow trout. Since the zebrafish and the rainbow trout UPIII are closely related to each other and seemed equally distant from UPIIIa and UPIIIb, we designated these genes proto-UPIII genes (Fig. 4B). The topology of the UPII, UPIIIa and UPIIIb tree (Fig. 4B) indicated that, the divergence of uroplakin Ia and Ib genes coincided with the appearance of uroplakins II and III genes, which have a similar intron/exon organization and a highly homologous exon in which a stretch of ~12 amino acids was shared by all UPII and UPIII proteins (Lin et al., 1994; Wu and Sun, 1993). Duplication of proto UPIII into UPIIIa and UPIIIb genes seemed to have happened later, before frogs and mammals diverged. Finally, a thorough search of the completed genome database of modern puffer fish (*F. rubripes* and *T. nigrovirides*), medaka (*O.latipes*) and sea squirt (*C. intestinalis*), a primitive urochordate, yielded many tetraspanin proteins, but no uroplakin-related sequences.

3.3. Co-evolution of the tetraspanin uroplakins and their associated proteins

To test the hypothesis that the genes encoding the tetraspanin UPIs may co-evolve with those encoding their major, tightly associated proteins, i.e., UP II and IIIa, we analyzed the sequence relationships in the four possible (tetraspanin/associated protein) pairs, i.e., UPIa/UPII,

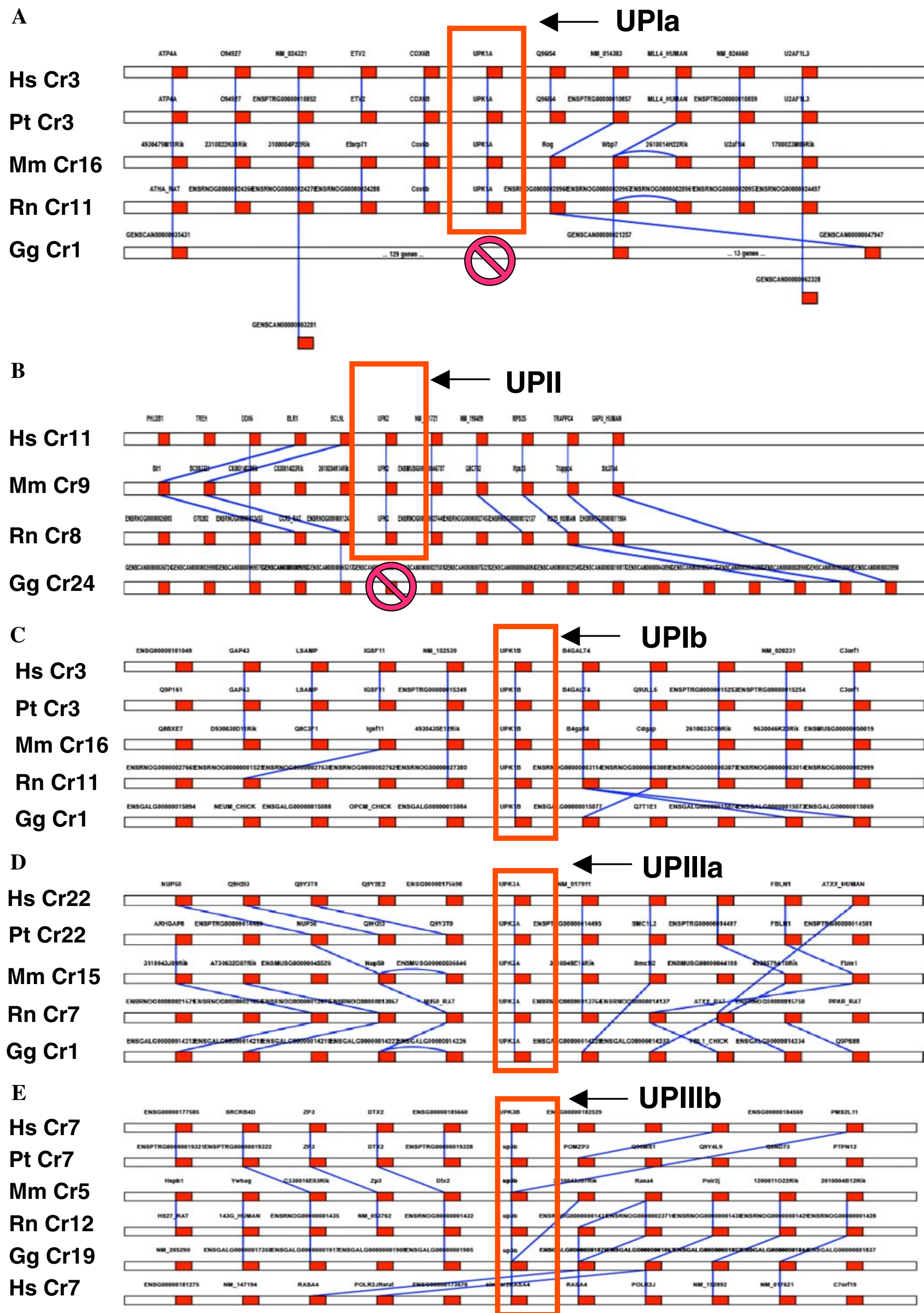

Fig. 3. The loss of uroplakin Ia and II genes in the chicken genome. (Absence of UPIa and UPII genes in the gallus genome (A and B). The location of UPIb, UPIIIa and IIIb genes (C, D and E)) Genes are highlighted in red and homologous genes are linked with blue lines. Five human genes on each side of the human uroplakin gene were used to blast against the genomes of chicken (Gg), mouse (Mm), rat (Rn), and chimpanzee (Pt). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

UPIa/UPIIIa, UPIb/UPII and UPIb/UPIIIa. Our analyses, shown in Fig. 5 as tanglegrams, addresses the significance of co-evolutionary relationships of the various pairs, and indicated a strong association between UPIa and UPII ($P < 0.0001$), and between UPIb and UPIIIa ($P < 0.0001$). The association between UPIa and UPIIIa was only moderately significant ($P < 0.05$), while that between UPIb/UPII was statistically insignificant (Fig. 5 and Supplementary Table 2). These results suggest that UPII co-evolved strictly with UPIa, while UPIIIa co-evolved strongly with UPIb. Further work examining the detailed interactions between these proteins will determine the mechanisms of these co-evolutionary relationships.

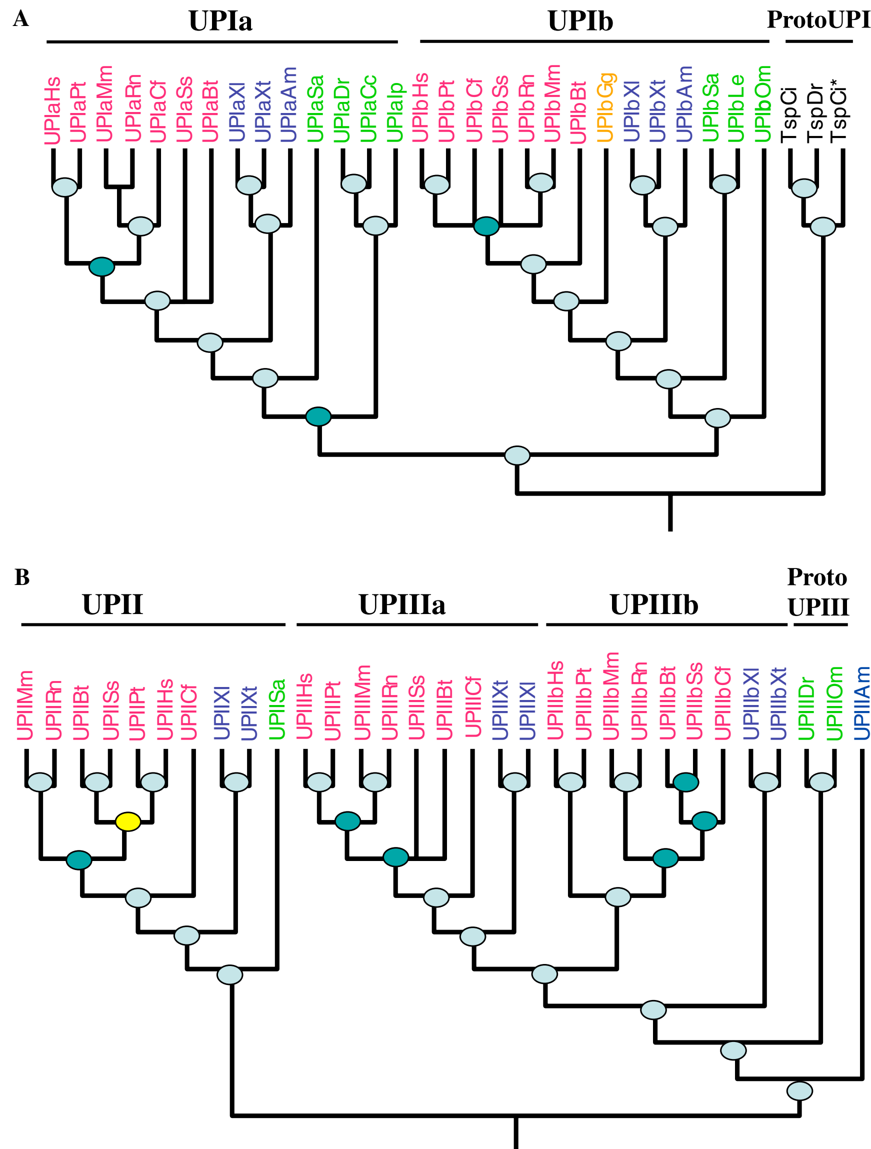

Fig. 4. The evolutionary relationship among animal proteins as depicted in genealogical trees. (A) Uroplakin Ia and Ib. (B) Uroplakins II, IIIa and IIIb. Colored circles indicate support values at nodes; light blue indicates greater than 90% bootstrap (Felsenstein, 1985) and jackknife (Farris et al., 1996) support and greater than 95% Bayes probability (Huelsenbeck and Ronquist, 2001); dark blue circles indicate 65–90% bootstrap and jackknife support and 90–95% Bayes probability; yellow circles indicate less than 65% bootstrap and jackknife support and less than 90% Bayes probability. Red labeled taxa are mammalian UPs (Hs, human; Pt, chimpanzee; Ss, pig; Bt, bovine; Cf, dog; Rn, rat and Mm, mouse), yellow taxa are birds (Gg, chiken), blue taxa are amphibian (Xt and Xl, frogs; Am, axolotl), green taxa are fish (cartilagenous fish: Sa, shark and Le, little skate; and bony fish: Dr, zebrafish; Ip, catfish; Cc, carp and Om, rainbow trout), and black are outgroup tetraspanins (Dr, zebrafish and Ci, sea squirt ciona). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

4. Discussion

Since urothelial plaques consisting of two-dimensional crystalline arrays of 16-nm uroplakin particles are not known to exist outside of the mammalian urothelium (Hicks, 1975; Wu et al., 1994), it is unexpected that uroplakin-related genes exist and are expressed in a wide range of vertebrates including chicken (Fig. 3), frog (Figs. 1 and 2) and zebrafish (Fig. 1). The analyses of these diverse uroplakin gene sequences provide new insights into how uroplakins may have evolved (Figs. 5 and 6), and broaden our view on the structure and function of this group of integral membrane proteins.

4.1. The origin and evolution of tetraspanin uroplakins

Phylogenetic analyses of the uroplakin-related DNA sequences from mammals, chicken, frog and fish allowed us to construct a gene genealogy of UPIa and UPIb genes (Fig. 4A), and of UPII and UPIII genes (Fig. 4B). These results infer a pattern of uroplakin gene evolution as shown in Fig. 6. An ancestral proto-uroplakin I gene

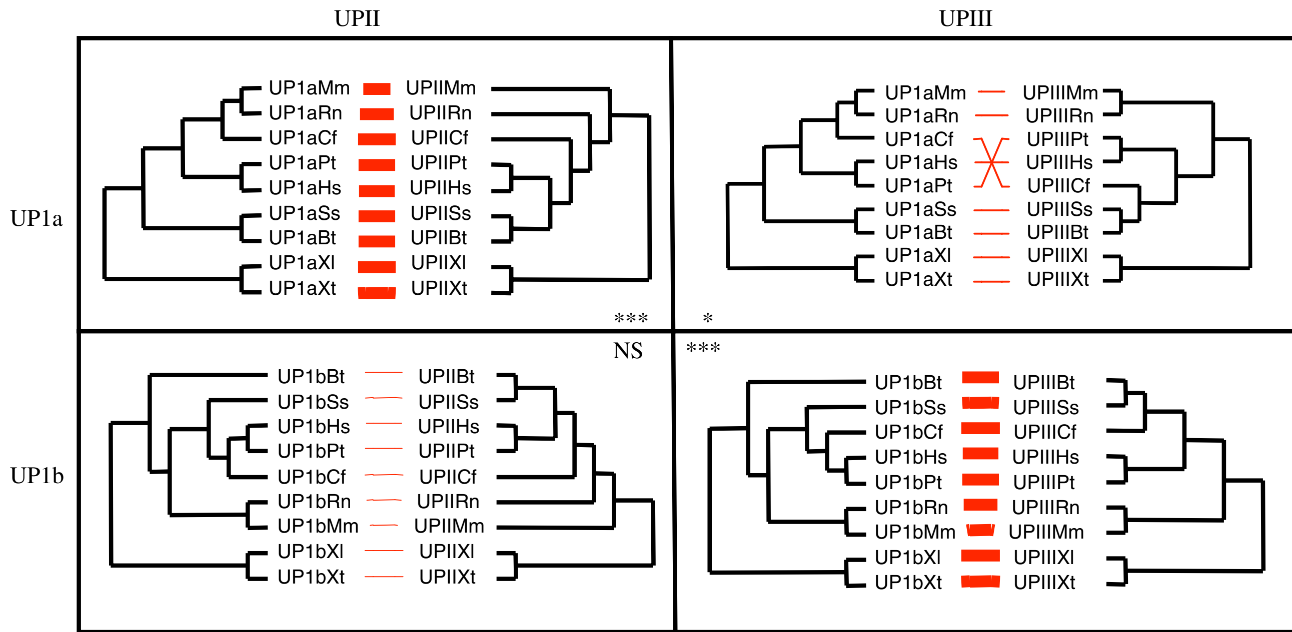

Fig. 5. UPIa and UPIb co-evolved with UPII and UPIIIa, respectively, as shown in a tanglegram. The reasoning for this approach is to first establish significant co-evolutionary relationships of the "paired" UPIa with UPII and UPIb with UPIIIa. As a control or contrast we also tested the significance of co-evolutionary relationships of the "non-intuitive" pairings of UPIa with UPIII and UPIb with UPII. Results of this approach are found in Table 1. The significance levels of the randomization tests implemented in TREEMAP 2.3 (Charleston and Page, 2002) are shown in the middle of the diagram. NS indicates not significant (for exact probability see Supplementary Table 2), * indicates significance at $P = 0.05$ and *** indicates significance at $P = 0.0001$.

duplicated in the common ancestor of vertebrates to become UPIa and UPIb, possibly with the concurrent formation and co-evolution of genes encoding UPII and UPIII (Figs. 5 and 6). These four major uroplakins Ia, Ib, II and III (IIIa and IIIb in tetrapods) are characteristic of, and are highly conserved in, all the present-day mammals (Wu et al., 1994). In addition, our results revealed several potential gene loss events: (i) the loss of the UPIa/II pair in birds as they evolved a drastically different body plan including their urinary tract system (Figs. 3 and 6); and (ii) the deletion in bony fish of the UPIb and UPII genes in zebrafish and the deletion of all uroplakins in puffer fish and medaka (Fig. 6). The evidence and arguments in support of these hypothetical events, and their possible significance, are discussed below.

4.2. Deletion of uroplakin genes in some species

Despite extensive searches of the genome database, we could not find any ortholog of the UP genes in puffer fish (*F. rubripes* and *T. nigroviridis*) and medaka (*O. latipes*), that are modern bony fish, although these orthologs were already present in the more primitive cartilaginous fish. It is well known that the genome of the common ancestor of teleost fish underwent a duplication event (Taylor et al., 2001, 2003). Our inability to retrieve UP sequences from teleost fish is somewhat inconsistent with this duplication event, indicating that the missing genes could be the result of actual elimination of the genes from the teleost genome or extreme divergence of the original orthologs such that data base searches do not detect them. In either case, our results, along with the potential genomic loss of UPIb and UPII genes in zebrafish, indicate that UP genes are, in some bony fish, dispensable, or can be functionally substituted by other genes. In the case of the chicken, although we readily detected the uroplakin Ib, IIIa and IIIb genes in the chicken genome, extensive search of the genome gave no trace of UPIa- and II-related sequences (Fig. 3). Given the fact that frogs, which are more primitive than chicken, have already acquired *all* the uroplakin genes, the most parsimonious explanation is that chicken has lost its UPIa/UPII genes during evolutionary divergence. The relatively small number of species that we have studied so far does not allow us to make a more definitive statement about such losses of uroplakin genes from genomes in higher vertebrates, but whole genome sequences of more birds and reptiles as well as more fish will help to address this point.

Although *Fugu* and *Tetraodon* have about the same number of genes as humans, their genomes are much smaller than that of humans because of the elimination of repetitive elements leading to a densely packed genome with short intergenic and intronic sequences (Aparicio et al., 2002). However, not all fishes of the Acanthopterygii superorder have compacted genomic. For example, the genome of medaka also lacks uroplakins, even though it contains about 1 billion base pairs (genomic sequence 91% completed), which is similar to the genome size of catfish. These results suggest that the elimination of uroplakin genes in *Fugu* and *Tetraodon* is not a general consequence of genomic compaction.

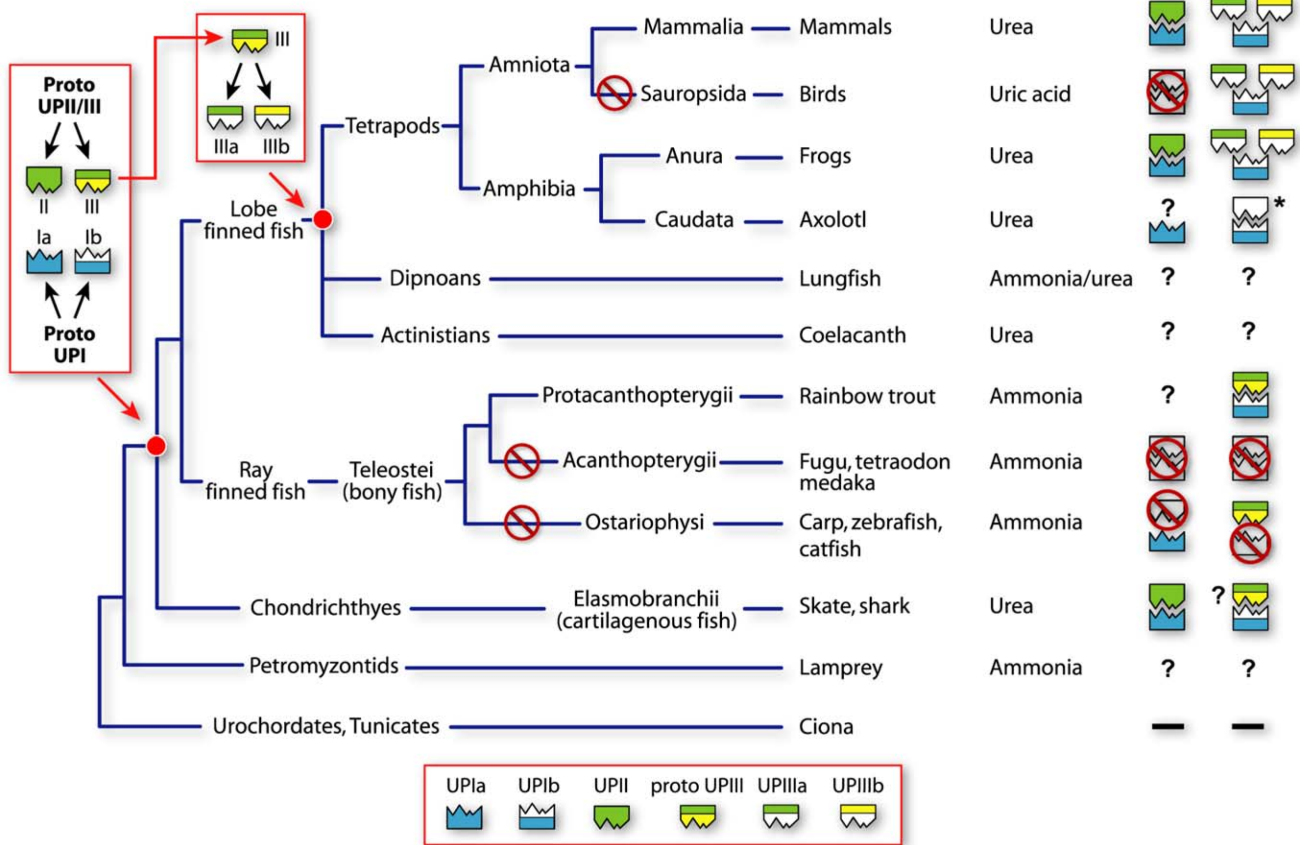

Fig. 6. A schematic model showing hypothesized events involved in the evolution of the uroplakin gene family. Significant evolutionary events in the divergence of uroplakins are shown by cartoons on lineages where events are hypothesized to have occurred. Duplications of the proto-UPI and proto UPII/III genes occurred in the ancestor of vertebrates. First UP sequences were found in cartilagenous fish. Losses of various UP genes are indicated by a red circle with a slash. The lack of UP-related genes in sea squirt ciona is indicated with a dash. A question mark denotes that although we have not found UP sequences, we could not rule out its presence because the corresponding EST data bases were small and/or genomic sequencing had not been completed. Asterisk denotes a truncated Axolotl UPIII protein. The predominant form of nitrogen waste, ammonia, urea or uric acid for each lineage is indicated. See text for discussion of these events.

4.3. Co-evolution of the tetraspanin uroplakin genes and their associated proteins

With the exception of *D. rerio*, which seems to possess an UPIa-like gene but lacks an UPII-related gene, the divergence of UPIa and UPIb genes in other vertebrate lineages coincides with the acquisition of genes encoding their respective associated proteins UPII and UPIIIa/b (Figs. 4 and 6). Moreover, analyses of the tetraspanin UPIa and UPIb with their major associated, single-transmembrane-domain, proteins UPII and UPIIIa by the tanglegram approach revealed that UPIa and UPIb co-evolved with UPII and UPIIIa, respectively (Fig. 5). The concomitant loss of UPIa and UPII genes in chicken (*G. gallus*; Fig. 3) provides additional, strong support for a tight co-evolutionary relationship between UPIa and UPII in tetrapods. Taken together, these results clearly establish that UPIa and UPIb genes, in the analyzed tetrapods, are co-evolving with UPII and UPIIIa genes, respectively. This co-evolutionary relationship is entirely consistent with the idea that UPIa and UPIb bind to UPII and UPIIIa, respectively, forming heterodimers UPIa/II and UPIb/III, as suggested by (i) our chemical crosslinking data showing the preferential formation of the UPIa/II and UPIb/IIIa heterodimers (Wu et al., 1995), (ii) the isolation of the UPIa/II and UPIb/IIIa complexes (Liang et al., 2001), (iii) the transfection studies showing that the formation of UPIa/II and UPIb/III heterodimers is a prerequisite for ER exit (Deng et al., 2002; Hu et al., 2005; Tu et al., 2002), and (iv) the mouse genetic ablation studies showing that the deletion of UPII or UPIIIa gene led to the mistargeting of their respective partner proteins (Hu et al., 2000; Kong et al., 2004). The analysis of the 'mismatched' pairs by the tanglegram showed that the co-evolution of the mismatched UPIa/UPIIIa and UPIb/UPII pairs was either not significant ($P > 0.3$) or only slightly significant ($P = 0.05$) compared to the other possible combinations. Structural analyses is needed to physically corroborate such relationships.

4.4. Changes in nitrogenous metabolism and uroplakin composition

What is the meaning of the surprisingly divergent uroplakin patterns observed in fish, frog, chicken and

mammals? These patterns may in part relate to the different strategies that vertebrates have developed to excrete their nitrogen wastes when they adapt to different habitats (Fig. 6) (Barimo et al., 2004; Evans et al., 2005; Kong et al., 1998; Mommsen and Walsh, 1989; Walsh, 1997; Wright, 1995). The main nitrogenous excretory product of aquatic vertebrates, with the exception of some cartilaginous fish and the coelacanth which excrete urea, is ammonia (ammonotelism), which is quite toxic; thus its excretion requires a large volume of water (Ip et al., 2004; Wright et al., 2004). While water is not a limiting factor for aquatic animals, tetrapods including amphibians and mammals evolved mechanisms to excrete mainly urea (ureotelism) that is metabolically more expensive requiring at least two extra ATPs per molecule to make, but it is highly soluble and relatively non-toxic, thus allowing the excretion of the nitrogenous wastes with a minimal water loss (Wilkie, 2002). Some fish, such as cartilagenous fish and the coelacanth, utilize urea for osmoregulation, as well as for nitrogen excretion; in these fish urea has to be kept from leaking through the membranes (Hill et al., 2004; Walsh, 1997). The metabolism of birds, lizards and snakes evolved to produce the highly insoluble uric acid (uricotelism) as their main nitrogenous excretory product primarily because of their use of a cleidoic egg in reproduction. In these species, the urine is directly discharged from the kidneys to the cloaca, which results from the fusion of the rectum and the ureter.

The urinary bladder mainly functions as a short-term storage site for urine (Lewis, 2000). Our results indicate that uroplakins are expressed mainly by ureogenic vertebrates (Fig. 6). This finding, coupled with our understanding that mammalian uroplakins contribute to the formation of the urothelial permeability barrier (Hu et al., 2000, 2002), suggests that uroplakin evolution is linked to the formation of a membrane capable of maintaining an osmotic urea gradient. Our data on a limited number of species thus raise the interesting possibility that uroplakins are associated with ureotelism and with the maintenance of elevated urea concentrations in some vertebrates. This idea is supported by our observation that some or all of the UP proteins can become dispensable when ureotelism changed to ammonotelism or uricotelism such as in bony fish and birds (Fig. 6). This result also suggests that the uroplakin trait, like limbs and lungs in lobe finned fish, is a critical adaptive step enabling some tetrapod ancestors to leave the aquatic environment and to become adapted to the new water-restricted terrestrial habitat. Additional studies on more species are needed to see whether such a correlation will hold, and, if so, to further define its functional basis.

4.5. *Frog vs. mammalian uroplakins*

Analyses of the *X. laevis* and *X. tropicalis* uroplakins revealed several interesting features. First, the frog bladder epithelium expresses the orthologs of all four major mammalian uroplakins (Ia, Ib, II and IIIa) as well as that of the minor mammalian UPIIIb (Fig. 2). An important function of frog bladder epithelium is water-absorption, while that of mammalian epithelium is the opposite, i.e., to form a permeability barrier (Hicks, 1975). Interestingly, frog bladder epithelium does not elaborate urothelial plaques similar to those of mammals. Although frog bladder epithelium does make some intra-membranous particles, these particles are structurally and functionally distinct from the uroplakin particles of mammalian urothelia. The frog particles are only 4.5–8.5-nm in diameter and are therefore much smaller than the 16-nm mammalian urothelial particles (Rash et al., 2004; van Hoek et al., 1998). They partition during freeze-fracture to the P face, whereas the uroplakin particles partition to the E face (Kachar et al., 1999; Staehelin et al., 1972; Vergara et al., 1969; Wade et al., 1975). Moreover, the particles on the apical surface of the frog bladder epithelium increase in number and in their degree of organization under conditions of enhanced water-absorption (Bourguet et al., 1976; Kachadorian et al., 1975), consistent with the suggestion that these aggregates contain the water channels (Sun et al., 2002). Therefore it seems likely that the frog uroplakin-orthologues, whose amino acid sequences are significantly different from those of the mammals (Fig. 1), have not yet acquired the capacity to form the 16-nm particles. This result suggests that uroplakins acquired the ability to form the 16-nm particles and 2D crystals only in the mammals. Alternatively, the frog uroplakins fail to form particles and crystals because of a lack of some other aspects of the bladder epithelial membranes, such as special lipids, that might be unique to the mammals. Second, a comparison of the frog uroplakin sequences with those of the human, bovine and mouse revealed that uroplakin Ia and Ib sequences are much more similar to their mammalian counterparts (average 60.6% and 72.7% identical to those of the mammalian orthologues, respectively) than those of the UPII (35.6%), UPIIIa (36.1%) and UPIIIb (37.1%; Fig. 1 and Table 1). These data suggest that the tetraspanin uroplakins probably have experienced more constraints, than their associated proteins, with evolutionary diversification of the vertebrates. Thus UPII, IIIa and IIIb have changed significantly during the amphibian-to-mammal transition; some of these changes may be responsible for the mammalian uroplakins' newly acquired ability to form particles and 2D crystals. Third, the frog orthologs of all four major mammalian uroplakins (Ia, Ib, II and IIIa) are co-expressed in large amounts in some non-urothelial cells including kidney, fat body and oocytes (Fig. 2; (Mahbub Hasan et al., 2005; Sakakibara et al., 2005)), and therefore appear to be far less "bladder-specific" than their mammalian counterparts (Deng et al., 2002; Lin et al., 1994; Wu and Sun, 1993; Yu et al., 1994). The structural and functional significance of the frog uroplakins in bladder and other non-bladder tissues is unknown. Sakakibara and coworkers have recently shown that a *Xenopus* oocyte protein, identified as uroplakin III,

becomes tyrosine-phosphorylated upon fertilization suggesting that this uroplakin may play a role, at least in this species, in early fertilization (Mahbub Hasan et al., 2005; Sakakibara et al., 2005). Fourth, the *Xenopus* ortholog of mammalian UPIIIb, which is a minor component of mammalian urothelial plaques (Deng et al., 2002), is the only uroplakin that seems to be relatively bladder-specific (Fig. 2). Additional studies are needed to better understand the structure and function of uroplakins in *Xenopus* bladder epithelium and other tissues.

Axolotl (*Ambystoma mexicanum*) is an aquatic salamander that remains neotenic for life. So far we have found only UPIa, UPIb and a seemingly mutated UPIII, but no UPII for this animal. Given the limited sequence data available for this species we predict that this species, like other amphibians, has all uroplakin genes. A comparison of the uroplakins of axolotl and its terrestrial relative *Ambystoma tigrinum* may provide unique opportunity for better understanding uroplakin function.

5. Concluding remarks

We have established that uroplakins evolved very early during vertebrate evolution, since orthologs of UPIa, UPIb, UPII and UPIII genes can be seen even in fish (Figs. 1, 4 and 6). Our data indicate that the appearance of the UPIa and UPIb genes coincides with the formation of genes encoding their associated proteins, UPII and UPIII, respectively (Fig. 6). Although the uroplakin sequences are quite conserved from fish to mammals (particularly within the mammals), the species- and tissue-specificities of uroplakins can be highly variable, as indicated by our findings that (i) ammonotelic bony fish can lose various UP proteins during evolutionary divergence from ureotelic cartilagenous fish and tetrapods, with the most extreme example being the modern pufferfish and medaka which have lost all their UP genes; (ii) the chicken has lost its UPIa/UPII genes, and (iii) uroplakins can express coordinately in *Xenopus* tissues outside of the urinary tract, including oocytes and fat body. These results indicate that uroplakins have an ancient origin, and that *mammalian* uroplakins have acquired certain features enabling them to form 16 nm particles and 2D crystals that contribute to the urothelial permeability barrier function. Overall, our results indicate that the structure and function of uroplakins are much more diverse and versatile than hitherto appreciated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.04.023.

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