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The Amphibian Diacylglycerol O-acyltransferase 2 (DGAT2): a 'paleo-protein' with Conserved Function but Unique Folding

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Abstract

Amphibians are, currently, considered the first vertebrates that had performed the aquatic to terrestrial transition during evolution; therefore, water balance and dehydration control were prerequisites for such environment conquering. Among anurans, *Phyllomedusa* is a well-studied genus, due to its peptide-rich skin secretion. Here, we have analyzed the skin secretion of *Phyllomedusa distincta* targeting the proteins present in the skin secretion. The major soluble protein was chromatographically isolated and utilized to immunize rabbits. Through proteomics approaches, we were able to identify such protein as being the diacylglycerol O-acyltransferase 2 (DGAT2), a crucial enzyme involved in lipid synthesis and in the skin water balance. Immunohistochemistry assays revealed the protein tissular distribution for different animal species, belonging to different branches of the phylogenetic tree. Specifically, there was positivity to the anti-DGAT2 on Amphibians' skin, and no antibody recognition on fish and mammals' skins. The DGAT2 multiple sequence alignment reveals some degree of conservation throughout the genera; however, there is a different cysteine pattern among them. Molecular modeling analyses corroborate that the different cysteine pattern leads to distinct 3D structures, explaining the different antibody recognition. Moreover, the protein phylogenetic analyses place the *Xenopus* DGAT2 (the available amphibian representative) next to the *Coelacanthus* enzyme, which have led the authors to term this a 'paleo-protein'. DGAT2 would be, therefore, an ancient protein, crucial to the terrestrial environment conquest, with a unique folding—as indicated by the molecular models and immunohistochemistry analyses—a consequence of the different cysteine pattern but with conserved biological function.

Keywords Amphibian · *Phyllomedusa distincta* · DGAT2 · Phylogeny

1 Introduction

The Class Amphibia currently comprises three orders (Anura, Caudata and Gymnophiona), which are classified together within the Lissamphibia [1], which also includes the extinct amphibians. Although the extant amphibians have usually been considered as a monophyletic group, some authors have been questioning this hypothesis and defend that anurans and salamanders are distant of caecilians and may have arisen separately from different ancestral Paleozoic amphibian groups [1–3]. However, the problem in establishing the main lines of amphibian phylogeny lies in the scarce fossil record, particularly for caecilians, which seems to be a key group to the better understanding of amphibian evolution. Moreover, the early history of modern amphibian groups has not been well understood as well, due to the fact that they possibly acquired their specialized morphology very early in their evolutionary

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histories [1, 4]. Particularly, living anurans and caecilians are highly modified animals in terms of anatomy: frogs and toads have adapted for jumping, whereas caecilians have entirely lost their limbs and have become primarily adapted to a fossorial lifestyle [5]. Because these groups are so anatomically specialized, many questions regarding the origins, relationships, and historical distribution of the lissamphibians remain unresolved [1]. Recent papers try to provide more reliable data around these questions using a combination of morphological and molecular data [6].

In spite of their morphological diversity, all amphibians present a highly active skin that takes part in the major biological processes related to homeostasis, such as breathing, water balance regulation, reproduction and defense against both predators and microorganisms. Morphological studies conducted for several species of amphibians have shown that, regardless of the level and kind of specialization, all animals present two basic types of skin glands: mucous and granular [7]. Several studies have assessed the contents of the two gland types through a histochemical and/or immunohistochemical perspective and the consensus points to the mucous glands secreting molecules (proteins, glycoproteins, proteoglycans and glycosaminoglycans) responsible for homeostasis maintenance (mainly lubrication/hydration), whereas the granular glands would secrete molecules used in defense against predators and microorganisms (peptides, alkaloids, steroids) [8–10].

On the other hand, many researchers have extensively profiled the skin secretions (or skin extracts, in a more old-fashioned way) searching for active molecules—especially antibiotics [11–13]—after Zasloff's description of magainine when noticing that post-operatory infections rarely occurred in Xenopus [14]. The many published works on the subject compile thousands of different active molecules that can be roughly grouped into antibiotic peptides, vasoactive peptides, bradykinin related peptides, cardiotonic alkaloids and steroids, and hallucinogenic alkaloids [15, 16]. Nevertheless, most of these papers focus either on the biological activity of one (or few) isolated molecule(s) or on the large cataloging of the secreted molecules based on one given high-throughput technique [17–20].

The chemical profiling of the amphibian skin secretion has recently begun to be assessed as a species characteristic—a phenotype—similar to classical anatomical features. Sciani et al. [10] have evaluated the presence of alkaloids and steroids in the skin secretion of Brazilian *Rhinella* and demonstrated that there is a 'metabolic fingerprint' that could differentiate the species. Using the same approach but with another goal, Jeckel et al. [21] were able to show that there is a relationship between age, gender and size and the skin secretion composition. It is worth to mention that, in spite of the protein profiles depicted by Sciani et al. [10], both studies rely on the low molecular mass components for proper grouping.

Amphibian proteins have been poorly studied throughout the years. Recently, Libério et at. [22] have described the presence of peptidases in the skin secretion of *Leptodactylus labyrinthicus*, but mainly by detection of the enzymatic activity. Conceição et al. [23], on the other hand, have detected both the presence of proteins in the skin secretion (by SDS-PAGE) and some enzymatic activities (by specific enzymatic assays). Nevertheless, most authors limited themselves to describe the presence of proteins in the skin secretion while studying other amphibian features [7, 24]. Moreover, due to the syncytial nature of the amphibian granular glands, at least in anurans [7, 25], some secreted proteins may not be actual toxins but cellular 'debris', including the many housekeeping proteins, cytoskeleton, metabolic/catabolic/anabolic pathways. This would explain, for instance, the high degree of similarity, observed for the proteins, among the different *Rhinella* species studied by Sciani et al. [10] despite presenting significantly different profiles of low-molecular-mass molecules.

In spite of all the technical improvements that the contemporaneous analytical biochemistry techniques have brought to Toxinology (for the study of venoms and glandular secretions in general) one key point still challenges researchers dealing with amphibian skin secretion composition: which molecule is actually synthesized in which type of gland? In order to address this subject, we have selected the major protein—as assessed by RP-HPLC—from the skin secretion of *P. distincta* and, through immunohistochemistry and proteomics, worked on its identification, possible function and glandular localization. After the identification of such protein (diacylglycerol O-acyltransferase 2-DGAT2), sequence alignment, protein features and phylogeny analyses propose a unique folding and an ancient origin for this triacylglycerol synthesis enzyme that has been termed by the authors a 'paleo-protein'.

2 Materials and Methods

2.1 Amphibian Skin Secretion Attainment

Adult specimens (n = 5, SVL = 5.23 ± 0.36 cm) of the tree-frog *P. distincta* were collected in Iporanga, São Paulo. The animals were brought to the animal facilities of the Laboratory of Cell Biology, at Instituto Butantan, maintained in plastic terraria and weekly fed on crickets and cockroaches. Cutaneous secretion was collected every 2 months.

Skin secretion of six *P. distincta* (Anura, Hylidae) was obtained by submerging the animal in a beaker containing deionized water, for 10 min, while gently mechanically stimulating the surface of the body. The resulting solution

containing the pool of the skin secretion of all individuals was lyophilized and kept at − 20 °C, until further processing. All applicable institutional and/or national guidelines for the care and use of animals were followed.

2.2 Protein Purification

The skin secretion solution (1 mg mL^{-1}) was processed by an Amicon filter (10 kDa cut-off) and washed with ultrapure water. The > 10 kDa fraction was then fractionated by reversed phase high performance liquid chromatography (RP-HPLC), using a Proeminence 20A binary system (Shimadzu, Japan), employing a C4 (ACE 4.6 × 100 mm) column. The sample was eluted from the column by a two-solvent system: (A) trifluoroacetic acid (TFA) 0.1% in ultrapure water and (B) 90% acetonitrile (ACN) in ultrapure water containing TFA 0.1%, with a 10–80% B linear gradient over 20 min. Photodiode array (PDA) detection was used, scanning from 200 to 500 nm. The peak of interest (largest) was manually collected.

2.3 Antibody Production

"In house" antibodies were developed as follows: two male New Zealand rabbits, around 3 kg, were immunized with the purified protein as follows: 1.5 mg of protein was dissolved in 750 µL PBS and emulsified with the same volume of Montanide ISA 50 (SEPPIC, France) as adjuvant. From this volume, for each rabbit, 0.6 mL were injected sub-cutaneously in three distinct spots (200 µg/spot). After 15 days, this process was repeated. To ensure higher titers, four boosters consisting of the same amount of protein dissolved in PBS were given, using the same time interval. Antibody titers were monitored by enzyme linked immunosorbent assays during the immunization process. One week after the last booster, the animals were euthanized and the blood was collected by cardiac puncture. The serum was separated and frozen until use.

For IgG purification, as a first step, 3 mL of the serum was delipidated by the addition of 3 mL of 1M $CaCl_2$ and 120 µL of 10% dextran sulfate. After 30 min incubation on ice, this mixture was centrifuged at 10,000 × *g* for 10 min and the supernatant was dialyzed overnight against PBS. This delipidated serum was then injected in a 5 mL protein A Sepharose column (GE healthcare) previously equilibrated with PBS. Elution was performed using a 1 mL/min flow rate. After elution of the unbound protein, the IgGs were eluted from the column with 100 mM glycine pH 3. One mL fractions were collected in tubes containing 100 µL 1 M Tris pH 8.5. The IgG containing fractions were pooled and dialyzed overnight against PBS (Supplemental material).

2.4 Affinity Column Confection and Purification

The purified IgGs were coupled to an NHS-Sepharose matrix (GE Healthcare) following the manufacturer instructions. Briefly, around 5 mg of purified IgG in PBS were incubated overnight with 1.5 mL of resin, previously washed with 1 mM HCl. The binding efficiency was estimated by measuring the 280 nm absorbance of the antibody solution before and after coupling. Remaining reactive sites of the resin were blocked by incubating the resin with 1 M Tris/150 mM NaCl pH 8 for 16 h. The resin was then packed in a Tricorn 5 × 20 mm column (GE healthcare).

After solubilization in 1 mL PBS pH 7.4, 700 µg of secretion were loaded on the affinity column, previously equilibrated with the same buffer with a 0.5/minute flow rate. After elution of the unbound fraction, the immunoreactive peak was recovered by switching the mobile phase to 100 mM glycine pH 3. One mL fractions were collected in tubes containing 100 µL 1 M Tris pH 8.5 and analyzed by 12% SDS-PAGE.

2.5 Proteomic Identification

An aliquot of the protein utilized for the immunization assays was applied in a 12% SDS-PAGE, under reducing conditions, according to Laemmli [26]. The band was excised and processed for proteomic analyses. Briefly, the band was destained with methanol containing 5% acetic acid (AA). The gel was then dehydrated with ACN and rehydrated with 50 mM ammonium bicarbonate and incubated overnight with trypsin (20 ng/µL). Peptides were then eluted from the gel with 5% formic acid (FA) solution followed by 50% ACN containing 5% FA.

The peptides were then loaded into a C18 column (Supelco, 2.1 × 50 mm, 100 Å), coupled to a Shimadzu ESI-IT-TOF LC-MS^n system (Shimadzu Co., Japan). A linear gradient of 0 to 40% solvent B_1 (A_1: 0.1% AA; B_1: 90% ACN containing 0.1% AA) was employed to elute the peptides, which were monitored by a PDA prior to mass spectrometry analyses.

The main instrument parameters were 4.5 kV interface voltage, 1.76 kV detector voltage and 200 °C capillary temperature, under positive ionization mode, in a full scan mode (50–2000 m/z) followed by data dependent acquisition (DDA) MS^2, under a 100–2000 m/z range obtained after CID (Ar) fragmentation. Data acquisition and processing were performed by the LCMSsolution software suite (Shimadzu Co, Japan),

For protein identification, processed converted spectra were analyzed and by MASCOT (Matrix Science Inc), using MS/MS Ion Search mode, with tolerance of 0.5 Da for MS and MS/MS and manually checked for accuracy and

correctness. The full NCBinr and Amphibia (taxid:8292) subset databases were selected for search.

2.6 Immunohistochemistry Assays

Skin samples of *P. distincta* were obtained and immediately fixed by immersion in paraformaldehyde in PBS buffer. After 48 h, they were dehydrated in ethanol series (70–100%) and embedded in paraffin. Transversal 4 µm sections were obtained with a Microm HM340 E® (Thermo Fisher Scientific, USA) microtome using disposable steel blades and placed on silanized glass slides.

Immunohistochemical reaction was performed using the antibody produced in rabbits with a protein purified by RP-HPLC. The sections were deparaffinized, rehydrated, and incubated in 6% aqueous hydrogen peroxide for 30 min to quench endogenous peroxidase activity. The slides were not submitted to antigen retrieval treatment. The sections were incubated with antibody at 1:1600 overnight at 4 °C. ADVANCE® HRP system (Dako, USA) was used to detect the antigen–antibody complexes. The specimens were counterstained with Mayer's hematoxylin, dehydrated, and mounted onto glass coverslips and xylene-based mounting medium. Non-immunized rabbit serum was used as a negative control.

Skin samples of *Phyllomedusa*, other amphibians and from different vertebrate species were prepared using the same histological and imunohistochemical methods described above. The following species were used: *P. burmeisteri* (Anura, Hylidae), *Pithecopus nordestinus* (Anura, Hylidae), *Hypsyboas raniceps* (Anura, Hylidae), *Leptodactylus* sp. (Anura, Leptodactylidae), *Rhinella marina* (Anura, Bufonidae), *Pipa carvalhoi* (Anura, Pipidae), *Dermatonotus muelleri* (Anura, Microhylidae), *Siphonops annulatus* (Gymnophiona, Shiphonopidae), *Salamandra salamandra* (Caudata, Salamandridae), *Potamotrygon falkneri* (Myliobatiformes. Potamotrygonidae), *Pterophyllum scalare* (Perciformes, Cichlidae), *Gallus gallus* (Galliformes. Phasianidae), *Amerotyphlops brongersmianus* (Squamata, Typhlopidae), *Mus musculus* (Rodentia, Muridae) and *Homo sapiens* (Primata, Hominidae).

Photomicrographs were obtained in an Olympus BX51® microscope coupled to an Olympus QColor 5® camera (Tokyo, Japan), using Image-Pro® Express software (Media Cybernetics, USA) for image capture.

2.7 Sequence Alignment, Structural Modeling and Phylogeny Analyses

Sequence alignment was performed at the UniProt site, using the embedded Clustal Omega tool [27–29] with default parameters. Structural modeling was performed at the SwissModel site [30–33], using the default parameters. Phylogeny analyses were performed by the MEGA suite, using the default parameters [34, 35].

The sequence alignments were performed in two distinct ways: for phylogeny, all diacylglycerol-transferase (DGAT2) deposited as of July 2016 were downloaded (188 sequences) into a single FASTA file. For structural features analyses, DGAT2 from *Xenopus* (Q6P342 and Q6PAZ3), *Homo* (Q96PD7), *Ratus* (Q5FVP8), *Mus* (Q9DCV3), *Gallus* (H9KZK2), *Anolis* (H9GIZ3), *Danio* (Q4V9F0) and *Callorhinchus* (V9L411) were selected according to the evolutionary relationships, species assayed and proposed rationale.

3 Results

3.1 Skin Secretion Characterization

P. distincta crude skin secretion is rich in proteins, within a wide range of molecular masses, as observed in Fig. 1 (lane "C"). The protein profiles before and after filtering (> 10 kDa) are similar, being the > 10 kDa more concentrated, as expected. In addition, the filtration was successful to remove the peptides, as observed in Fig. 1 (lane < 10 k),

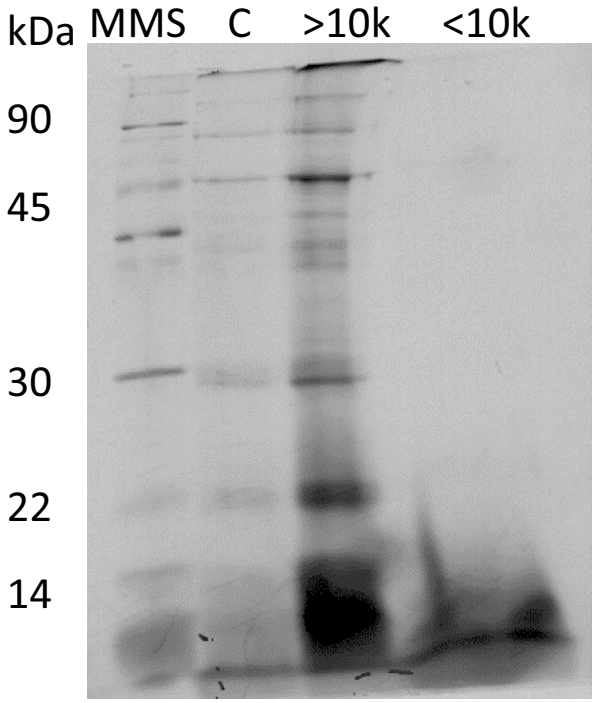

Fig. 1 SDS-PAGE 12%, stained by Coomassie Brilliant blue, of the *P. distincta* skin secretion MMS = molecular mass standard; C = crude secretion; >10 k = sample retained in the ultramolecular filter; <10 k = sample filtered in the ultramolecular filter

in which a band is visible in the lower end of the gel, correspondent to the many filtered peptides.

3.2 Protein Purification

The > 10 kDa fraction was then fractionated by RP-HPLC in a C4 column, yielding the chromatogram depicted in Fig. 2. The largest peak (arrow) was selected for manual collection and subsequent immunohistochemical analyses. The SDS-PAGE of the selected peak is presented in the inset in Fig. 2, lane 3 (arrow).

The protein could also be purified by immunoaffinity column, prepared with the antibodies produced in rabbits, after injection of *P. distincta* protein. This result confirmed the successful protein purification and specificity of the antibody (supplemental material—Fig. 1).

3.3 Proteomic Identification

The protein fractionated by C4 RP-HPLC was characterized by mass spectrometry, following the bottom up approach, e.g., the protein band from the SDS-PAGE was excised (Fig. 2, inset, lane 3), processed and analyzed by LC-MS/MS. The spectra were converted into MGF files and analyzed by MASCOT MS/MS ion search. Diacylglycerol O-acetyltransferase 2 (Q6P342), from *Xenopus tropicalis* was identified based on seven peptides, with a score of 42 and 8% coverage, as presented in Table 1.

3.4 Immunohistochemistry Assays

Purified Diacylglycerol O-acetyltransferase 2 (DGAT2), obtained from *P. distincta*, was injected in rabbits in order to obtain antibodies, to be used on immunohistochemical

Table 1 Peptides matching Diacylglycerol O-acetyltransferase 2 (Q6P342) after the proteomic processing of the RP-HPLC purified protein from *P. distincta* skin secretion

Start	End	Mr(expt)	Mr(calc)	Delta	Peptide
97	101	633.2427	633.3235	−0.0807	R.SSWVR.N
117	119	358.0127	358.2580	−0.2453	K.LVK.T
174	178	614.1427	614.3574	−0.2147	R.MPVLR.E
233	237	577.4827	577.3588	0.1239	R.KGFVK.V
271	274	573.4727	573.3275	0.1453	R.WIQK.K
275	278	549.6627	549.3275	0.3353	K.KFQK.Y
342	345	521.6427	521.2849	0.3578	R.LFDK.Y

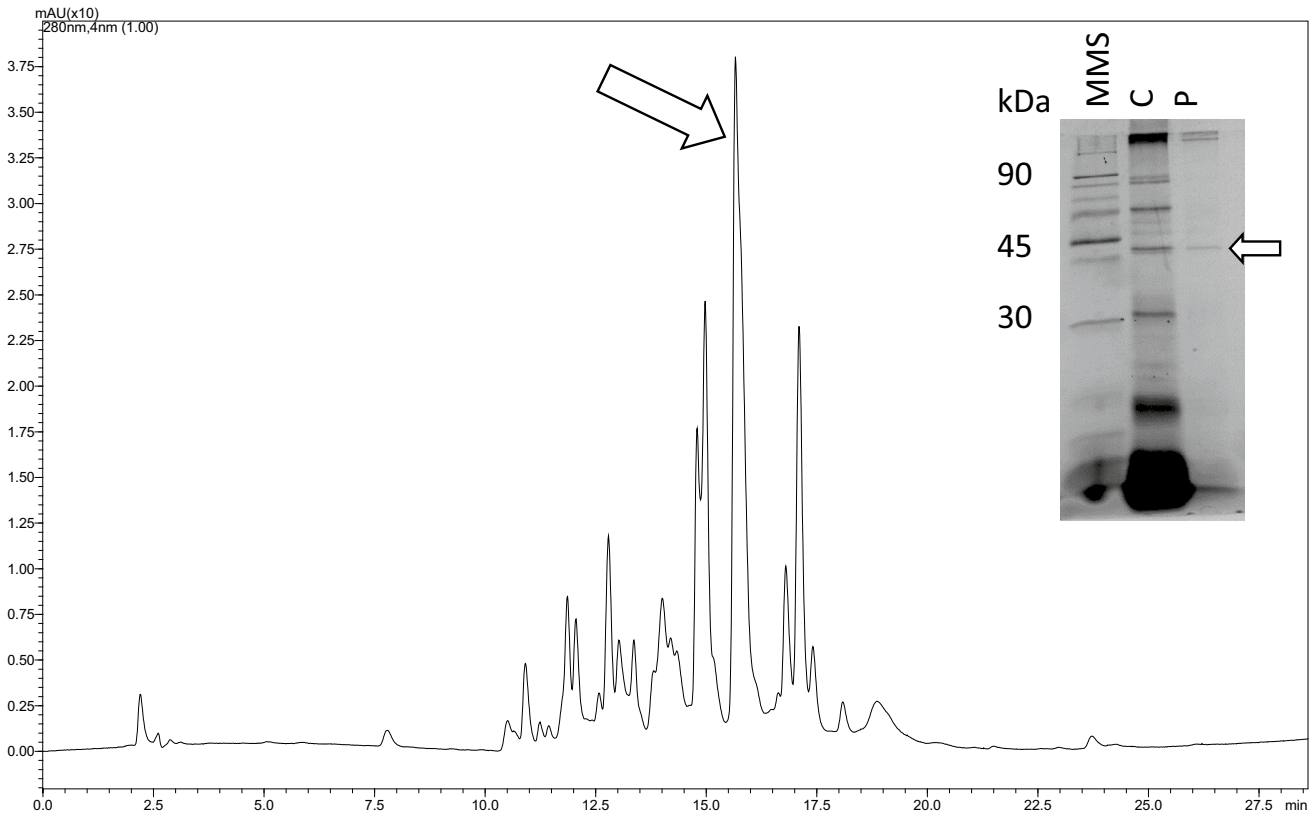

Fig. 2 C4-RP-HPLC profile of *P. distincta* skin secretion. The arrow shows the selected peak/protein for the immunoassay. Inset: SDS-PAGE of the crude venom (lane C) and the purified peak (lane P; arrow). MMS: molecular mass standard

assays. Sections of skin from several animal species were analyzed after incubation with anti-DGAT2: tree-frog, toad, frog, salamander, fish, snake, bird, mouse and human. Although the highest specificity was observed for genus *Phyllomedusa*, a positive reaction (represented by a brownish staining, derived from the peroxidase reaction, supplementary Fig. 2b) between protein and antibody could be observed in all tested amphibian species, although in different levels and in different skin tissues. Other vertebrates presented none or much less intense or specific immunoreactivity (Fig. 3). In all tested *Phyllomedusa* species (data presented only for *P. distinta* (A) *and P. nordestinus* (B)), the reaction was positive in the epidermal surface, in the lipid glands, in specific cells of the mucous glands, in the periphery of the poison glands and in the blood vessels. In the toad *R. marina* (D) and the pipid *P. carvalhoi* (F), some reaction was present in the poison and mucous glands, in the superficial layers of the epidermis and in the dermis. In the tree-frog *H. raniceps* (C), the reaction was detected mainly around the glands, in the myoepithelial layer, and in the dermal stratum spongiosum. For other amphibians, such as the frog *Leptodactylus* sp (E), the microhylid *D. muelleri* (G) and the caecilian *S. annulatus* (H), a strong reaction was observed throughout the dermal stratum spongiosum. In the salamander *S. salamandra* (I), the poison glands were especially reactive, and a scattered reaction in the epidermis and dermis was also observed. In the stingray *P. falkneri* (J) and in the bone fish *P. scalare* (K), no significant reaction was found. In bird (*G. gallus*, (L)), the epidermis presented some degree of reaction. The same was seen in the snake *A. brongersmianus* (M), in which the whole epidermis showed reaction. In relation to mammals, both mouse (N) and human (O) did not showed positive reaction. All the controls are presented in the supplemental material—Fig. 2a.

3.5 Sequence Alignment, Structural Modeling and Phylogeny Analyses

188 DAGT2 sequences were aligned by MEGA7 and this alignment was used to construct a phylogeny tree. Moreover, a restricted dataset of DGAT2 sequences was also used to build another alignment based on the actual immunohistochemistry data. Although the exact same species were not available in the UniProt database, we chose *Xenopus, Homo, Ratus, Mus, Gallus, Anolis, Danio* and *Callorhinchus* DGAT2 sequences to be aligned for their resemblance to the actual assessed tissues (Fig. 4). The restricted alignment and the phylogeny tree are present in Figs. 4 and 5 (and supplemental material—Fig. 3). It is interesting to note that in the smaller data alignment, the *Xenopus* DAGT2 sequences do not lie close to the *Anolis* protein, it is much closer to the fish (*Danio*) enzyme, whereas the *Anolis* enzyme is neighbor to the chicken protein. In the phylogeny analyses, this feature is even more exacerbated (Fig. 5): the closest neighbor (arrow in Fig. 5) to the branch in which the *Xenopus* enzymes (Q6P342 and Q6PAZ3) are located is H3AGL5, a DAGT2 from *Latimeria chalumnae* (West Indian ocean coelacanth). The other neighbor (A0A151M402) is an alligator enzyme (*Alligator mississippiensis*), but it is farther. There is no other amphibian or snake DGAT2 present in the closer branches, only fish and birds enzymes.

No vertebrate DGAT2 possess a published 3D structure; therefore, molecular modeling at the SwissModel was performed and the resulting structures of the *Xenopus*, ghostshark, human and mouse proteins are presented in Fig. 6. It is very interesting to note the similarities between the *Xenopus* and the ghostshark models that are very different from the human and mouse models (which are very similar one to another). The mammal's enzymes models propose a more compact, globular protein, whereas the *Xenopus* model reveals a protein with extended loops and a very different folding.

4 Discussion

Triglyceride synthesis is considered a key step in the formation of the adipose tissue, being the triacylglycerols accumulated in several tissues as an energy source [34]. The diglyceride acyltransferase (or O-acyltransferase), DGAT, is a transmembrane enzyme involved in the final step of the triglycerides formation, catalyzing the acylation of sn-1,2-diacylglycerol (DAG) at the sn-3 position using an acyl-CoA substrate.

The DGAT is homologous to other membrane-bound O-acyltransferases (MBOATs), enzymes that catalyze the same reaction, i.e. the transfer of an acyl group from acyl-coenzyme to one of several different substrates. The MBOATs are divided into three subgroups, according to the catalyzed reaction: (1) neutral lipid biosynthesis; (2) protein/peptide acylation and (3) phospholipid re-modelling [36].

There are different subtypes of DGAT, found in several species. The DGAT1 and DGAT2 are the most important ones, being responsible for the main part of triacylglycerols synthesis. These two enzymes are well known in eukaryotic organisms, represented as different genes families (*dgat1* and *dgat2)* with slight modifications on molecular and structural aspects, but with convergent function. (catalysis of triglyceride synthesis) [37].

In knockout studies, mice had either *dgat1* or *dgat2* genes silenced. Surprisingly, DGAT1−/− mice [38] were healthy and fertile and did not present any alteration in triglyceride levels. Moreover, the knocked-out animals were lean and seemed resistant to diet-induced obesity, which has called out the attention to the development of DGAT1 inhibitors focused on obesity treatment/control. On the

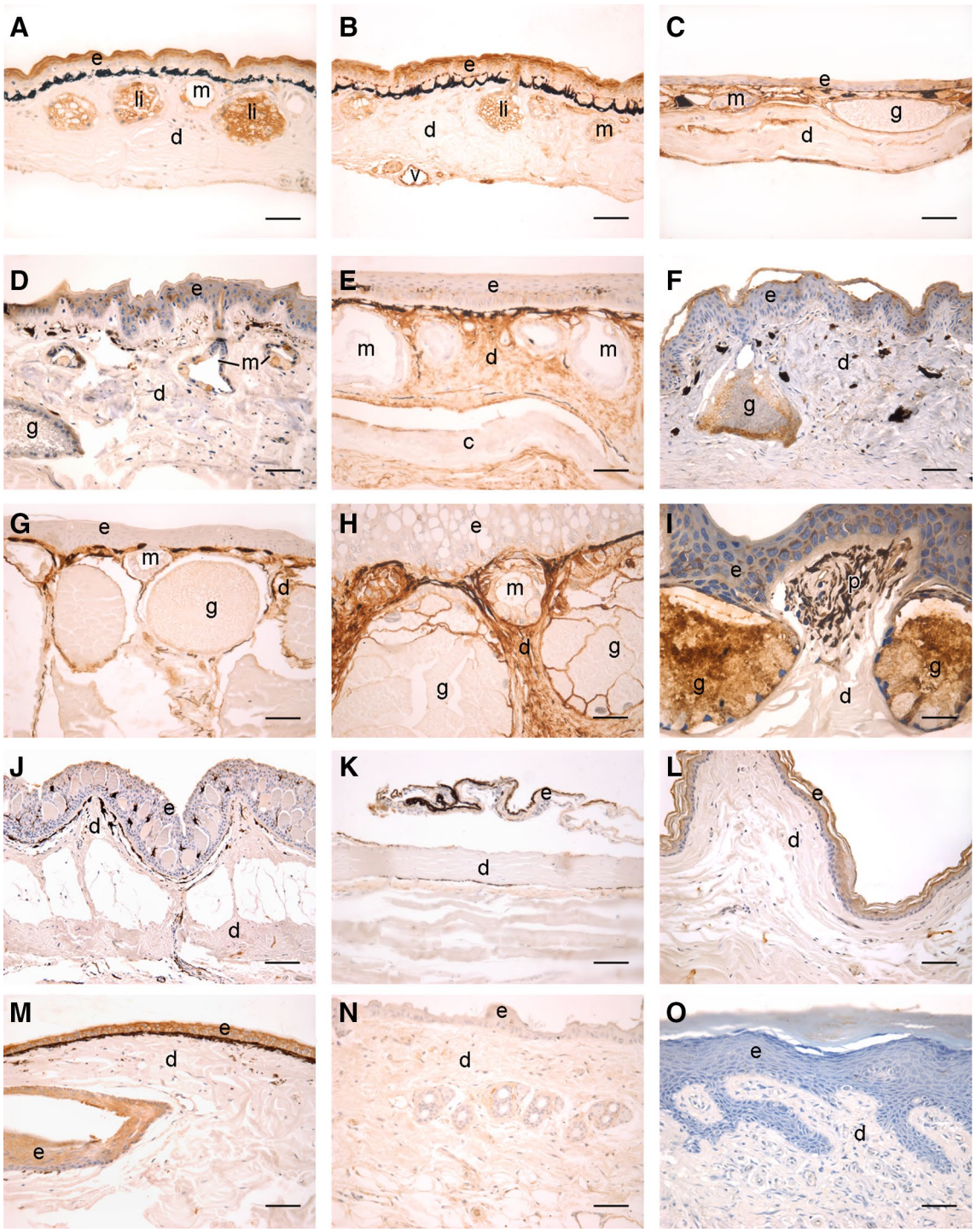

Fig. 3 Immunohistochemistry indicating DGAT2 localization in the skin of different species of amphibians (**a**–**i**) and of other vertebrates (j–**o**) **a** *Phyllomedusa distincta*; **b** *Pithecopus nordestinus*; **c** *Hypsiboas raniceps*; **d** *Rhinella marina*; **e** *Leptodactylus* sp; **f** *Pipa carvalhoi*; **g** *Dermatonotus muelleri*; **h** *Siphonops annulatus*; **i** *Salamandra salamandra*; **j** *Potamotrygon falkneri*; **k** *Pterophyllum scalare*; **l** *Gallus gallus*; **m** *Amerotyphlops brogersmianus*; **n** *Mus musculus*; **o** *Homo sapiens*. c, calcified dermal layer; d, dermis; e, epidermis; g, granular gland; li, lipid gland; m; mucous gland; v; blood vessel Bars = 50 µm

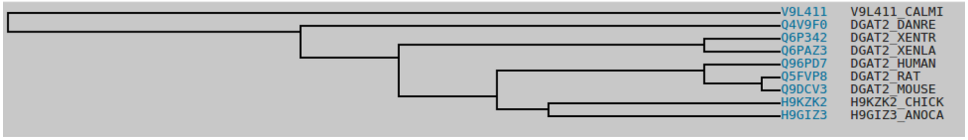

Fig. 4 Phylogeny of the alignment of DGAT2 sequences from database available representative species related to those evaluated in the immunohistochemistry assays, by antibody produced against *P. distincta* DGAT2. The full alignment is presented in supplemental Fig. 3. V9L411_CAMI: *Callorhinchus milii* (Ghost shark) DGAT2_DANRE: *Danio rerio* (Zebrafish) (*Brachydanio rerio*); DGAT2_XENTR: *Xenopus tropicalis* (Western clawed frog) (*Silurana tropicalis*); DGAT2_XENLA: *Xenopus laevis* (African clawed frog); DGAT2_HUMAN: *Homo sapiens* (Human); DGAT2_RAT: *Rattus norvegicus* (Rat); DGAT2_MOUSE: *Mus musculus* (Mouse); H9KZK2_CHICK: *Gallus gallus* (Chicken); H9GIZ3_ANOCA: *Anolis carolinensis* (Green anole) (American chameleon)

other hand, DGAT2−/− mice showed reduced triglycerides levels and lipopenia, and suffered from skin barrier abnormalities, including the inability to retain moisture, which led to death shortly after birth [39].

Amphibians are, currently, considered the first group of vertebrates that had performed the aquatic to terrestrial transition. Due to this evolutionary event, the Amphibians hydric balance is a particular phenomenon, dependent on morphological, physiological and behavioral adaptations, extremely important for their lives [40]. Thus, the dehydration control was a prerequisite for the terrestrial environment conquest. The conditions which favored the evolution of amphibians from fish, at the end of the Devonian period (around 380 mya) are still not entirely known. From the current fossil evidence, it is believed that representatives of freshwater lobed-fin fish, migrating from pond to pond during the dry seasons gradually increased their ability to stay on land during long periods of time [41]. The earliest amphibians originated from these fish gradually changed their morphology and physiology in order to face the new challenges imposed by their terrestrial life. Modifications able to surpass problems generated by dehydration were crucial during the evolution of these animals [40]. The integument is extremely important in this context, since it directly interacts with biotic and abiotic environmental factors, serving as a controlled barrier to the inward/outward water flow from the amphibian body [42].

Thus, water balance is one of the many crucial roles performed by the amphibian permeable skin [40, 43], which is intimately related to exchange of water, ions and respiratory gases between the animal and the environment. Reports describe the importance of lipids in the control of temperature and water loss, being a "specialized skin", resistant to water evaporation [44]. Recently we identified fatty acids in the skin of *Bokermannohyla alvarengai*, a peculiar tree frog that is able to bask in the sun as part of an efficient thermoregulatory strategy for survival in the dry, cold environment they inhabit. In this species, we identified the presence of lipids in their skin secretion, forming a waterproof layer on the skin surface [45].

The clear presence of DGAT2 in a group of vertebrates as dependent of water - as the amphibians are—is a strong indicative of the relationship of this enzyme with moisture retention. On the other hand, the differences in enzyme localization among the tested amphibian orders and species can be explained by the large variety of life modes among these animals. *Phyllomedusa* is one of the rare genera of amphibians that count with a quite impermeable skin due to the presence of specialized skin glands responsible for the secretion of lipid substances. By the use of the limbs, these substances are frequently spread throughout the body surface, in a behavior known as wiping behavior [46]. These characteristics can clearly explain the marked immune reaction obtained both in the lipid glands and on the surface of the epidermis (Fig. 3). In other amphibians, besides some reaction found in the skin glands, a strong reaction was seen throughout the dermis, which can constitute another type of barrier against water loss. Moreover, the lack of antibody recognition for the mammals does not imply the absence of the enzyme—which would be incompatible with life, according to Stone et al. (2004) [39]—rather, it is a clear indication of a different spatial structure, impairing antigen recognition, despite the fact that the amino acid sequences do not differ significantly (Fig. 3, supplemental material), but they do show a *different* cysteine pattern (Fig. 3, supplemental material, boxed regions), which usually bring forth an alternate folding.

It is important to mention that the protein models that are presented in Fig. 6 are not meant to be definitive. Rather, they are presented as an indication of the likely different folding of the proteins analyzed. The models were constructed by the automated SwissModel server using all default parameters under the following rationale: regardless of the true/actual structure of the enzyme, if SwissModel builds similar structures for 'different' protein sequences (e.g. mouse and human), those proteins ought to have actual similar 3D structures; however, if SwissModel—using the same parameters - build different structures for 'similar' protein sequences (mouse and *Xenopus*), these molecules should have actual different 3D structures.

Fig. 5 Phylogenetic tree of the 188 DGAT2 sequences deposited in the UniProt (Jan, 2018). The arrow indicates the *Xenopus* proteins. The bracket indicates the proximity to the coelacanthus enzyme

Based on this interpretation of the data, associated to an alternate cysteine pattern, we propose that the amphibian DAGT2 possess a unique folding, which is different from the other vertebrates. Moreover, combined analyses of the immunohistochemistry (Fig. 3) and phylogeny data (Fig. 5) of this enzyme glimpses on the unique structural features of the amphibian DGTA2 that makes this enzyme a unique metabolic 'fossil' record (neighbor only to the coelacanth, Fig. 5) of the fundamental lipid metabolism that grants the amphibian the necessary physical barrier that aids in the water balance. *Id est*, a paleo-protein as we propose.

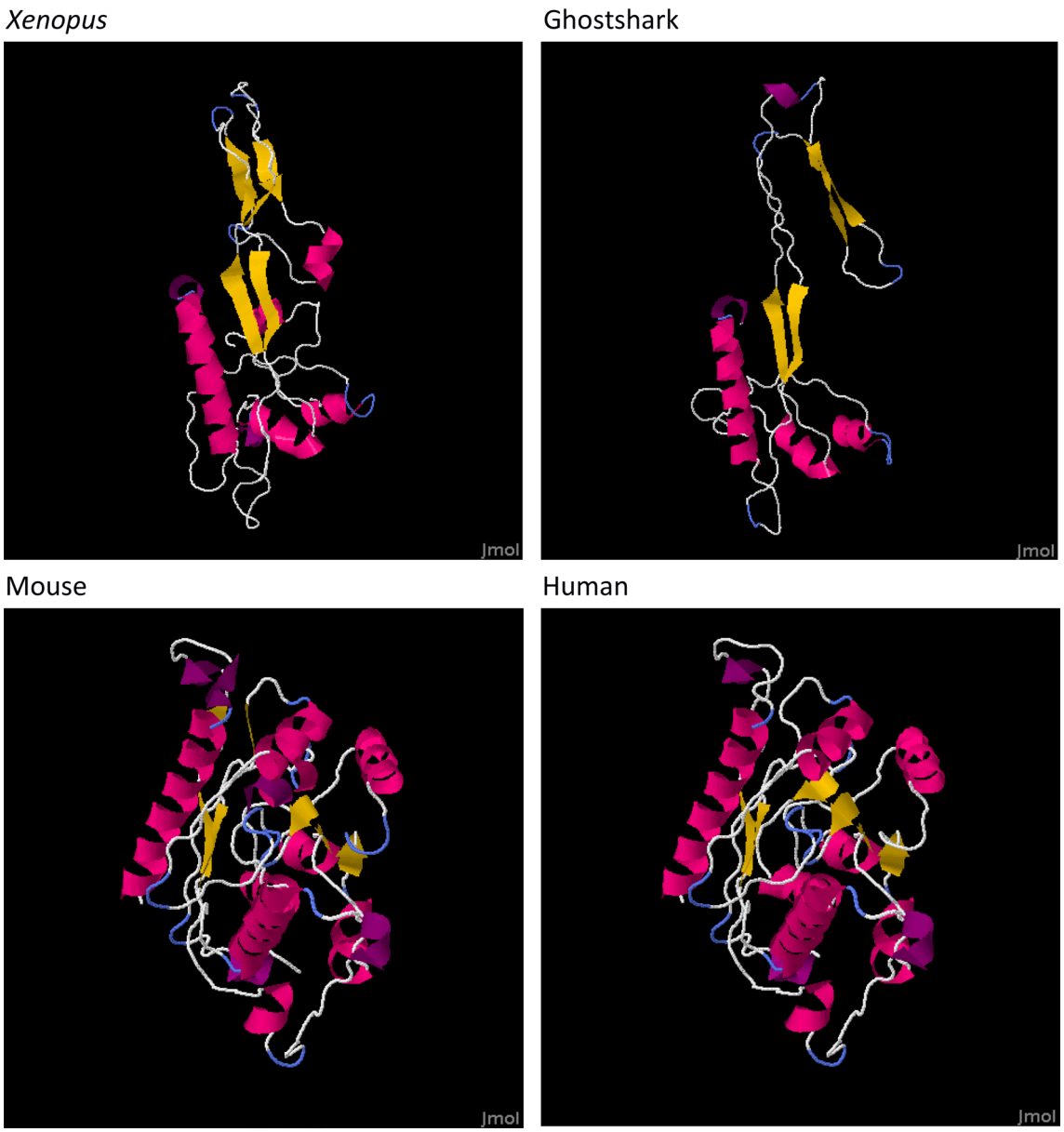

Fig. 6 DGAT2 3D models from *Xenopus*, ghostshark, mouse and human evincing the similarities in the structure between the mammal proteins and the differences between the amphibian and fish protein models

In conclusion, we have identified the actual tissular origin of the major protein secreted on the skin of *P. distincta* through immunohistochemistry assays. Moreover, by using a proteomic approach, we have actually identified the protein: DAGT2. Since this enzyme is involved in a very primordial biosynthetic pathway, we have tested antibodies developed by us against other species and found out that, in spite of the function conservation, profound differences in the antibody recognition could be observed. Therefore, we have performed more detailed protein sequence analyses and were able to observe that the cysteine pattern was not conserved throughout the species. Once this amino acid is critical for secondary and tertiary interactions within the protein, we have performed structural analyses of the modeled structures and were able to perceive that there are sound differences in the proposed structures among the proteins that would explain the segregated antibody recognition patterns. Altogether, the amphibian DAGT2 seems to be structurally unique and biologically primal for these animals: a paleoprotein that might be used in future studies as a key feature for proper biological relationship evaluations.

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Compliance with Ethical Standards

Conflict of interest Authors declare that they have no conflict of interest.

Ethical Approval The tree frogs were collected according to the Brazilian environmental agency (Brazilian Institute of Environment and Renewable Natural Resources—IBAMA) 17242-3. All animal procedures (including secretion attainment and histological experiments) were performed in accordance with the standards of the Ethics Committee on Animal Use of Instituto Butantan (CEUAIB) (protocol # 9532050216).

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