S-Palmitoylation and S-Oleoylation of Rabbit and Pig Sarcolipin*

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Background: Many functions are subjected to regulation by protein-protein interactions. An example is the SERCA1a-SLN complex.

Results: Rabbit and pig SLN have two types of fatty acid anchors (palmitic and oleic acid) attached to an intramembranous cysteine residue.

Conclusion: A role and evolutionary significance of these S-acylations are suggested.

Significance: First demonstration of SLN S-acylation and of the intramembranous S-oleoylation of a membrane protein.

Sarcolipin (SLN) is a regulatory peptide present in sarcoplasmic reticulum (SR) from skeletal muscle of animals. We find that native rabbit SLN is modified by a fatty acid anchor on Cys-9 with a palmitic acid in about 60% and, surprisingly, an oleic acid in the remaining 40%. SLN used for co-crystallization with SERCA1a (Winther, A. M., Bublitz, M., Karlsen, J. L., Møller, J. V., Hansen, J. B., Nissen, P., and Buch-Pedersen, M. J. (2013) Nature 495, 265–269; Ref. 1) is also palmitoylated/oleoylated, but is not visible in crystal structures, probably due to disorder. Treatment with 1 M hydroxylamine for 1 h removes the fatty acids from a majority of the SLN pool. This treatment did not modify the SERCA1a affinity for Ca$^{2+}$ but increased the Ca$^{2+}$-dependent ATPase activity of SR membranes indicating that the S-acylation of SLN or of other proteins is required for this effect on SERCA1a. Pig SLN is also fully palmitoylated/oleoylated on its Cys-9 residue, but in a reverse ratio of about 40/60. An alignment of 67 SLN sequences from the protein databases shows that 19 of them contain a cysteine and the rest a phenylalanine at position 9. Based on a cladogram, we postulate that the mutation from phenylalanine to cysteine in some species is the result of an evolutionary convergence. We suggest that, besides phosphorylation, S-acylation/deacylation also regulates SLN activity.
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As “one volume.” Depalmitoylation can be achieved by addition of Ca\(^{2+}\) transport (i.e. by the formation of a futile cycle; Refs. 7–9, 20), but also the role of the ryanodine receptor 1 Ca\(^{2+}\) channel must be considered in thermogenesis (19, 21).

In the present report, we demonstrate that rabbit and pig SLNs are fully palmitoylated or oleoylated at a membrane-embedded Cys-9 residue. Oleoylation of a cysteine residue appears to be a novel post-translational modification for a membrane protein. Hydroxylamine treatment of sarcoplasmic reticulum (SR), which leads to partial removal of these fatty acids, increases Ca\(^{2+}\)-dependent ATPase activity. We discuss our observations with respect to recently determined crystal structures of the SERCA1a-SLN complex and analyze phylogenetic data on SLN.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Octaethylene glycol mono-n-dodecyl ether (C\(_{12}\)E\(_{8}\)) was purchased from Nikkol Chemical (Tokyo, Japan), and n-dodecyl-\(\beta\)-d-maltopyranoside (DDM) from Affymetrix (Anatrace detergents and lipids, United Kingdom). TSK3000SW silica gel column was purchased from Tosoh Biosciences (Tessenderlo, Belgium). Most of the other chemical products were purchased from Sigma-Aldrich; see below for possible exceptions.

**Membrane Preparations**—Ca\(^{2+}\)-transporting sarcoplasmic reticulum (SR) vesicles were isolated from rabbit skeletal muscle according to published procedures (22, 23). From these preparations, purified Ca\(^{2+}\)-ATPase membranes were obtained by extraction of extrinsic proteins with a low concentration of deoxycholate (DOC-extracted SR; Refs. 24). Ca\(^{2+}\)-transporting sarcoplasmic reticulum (SR) vesicles were also isolated from pig and rat back skeletal muscle according to published procedures (22), except that the final supernatant after removal of large aggregates was carefully recovered, transferred into ultracentrifugation tubes in the presence of 2 mM KCl and 100 mM MgATP, and centrifuged for 90 min at 60,000 \(\times g\) (45Ti Beckman Coulter) to achieve extrinsic proteins removal and SR vesicles recovery. As for rabbit SR vesicles, the final pellet containing pig or rat SR vesicles was resuspended in 0.3 M sucrose for long-time storage at \(-80^\circ C\).

**Solubilization and Hydroxylamine Treatment**—SR vesicles were suspended at a concentration of 4 mg protein/ml\(^{-1}\) in the appropriate buffer (see figure legends for details, buffer A: 50 mM MOPS-Tris, pH 7.5, 25 mM NaCl, 1 mM MgCl\(_2\), and 1 mM CaCl\(_2\); buffer B: 50 mM MOPS-Tris, pH7.5, 25 mM NaCl, 50 mM MgCl\(_2\) supplemented with 40 mg/ml\(^{-1}\) DDM or C\(_{12}\)E\(_{8}\) for solubilization (detergent:protein ratio 10:1 w/w; Ref. 25), and incubated for 10 min at 20°C. Volume of the sample here is defined as “one volume.” Depalmitoylation can be achieved by addition of one volume of a freshly prepared 2 M hydroxylamine solution (buffered at pH 7.5 with saturated Tris) and incubation for 1 h at 20°C. For untreated “control” sample, one volume of initial buffer (or 2 M Tris-Cl at pH 7.5) was added instead of hydroxylamine. Hydroxylamine treatments were performed under various conditions: (i) on the detergent-solubilized and SEC-purified enzyme: the ATPase was first solubilized, injected on the column equilibrated with a buffer containing 50 mM MOPS-Tris pH7.5, 100 mM NaCl, 0.4 mg/ml\(^{-1}\) DDM, selected elution fractions were treated with hydroxylamine and finally submitted to a desalting step to decrease the amount of NaCl and hydroxylamine, which may prevent efficient analysis of the samples by MALDI-TOF mass spectrometry; (ii) in treating the native or solubilized ATPase before SEC purification and to purify the complex on a column initially equilibrated with the above buffer but with only 25 mM NaCl. This allowed us to omit the additional desalting step which was time consuming and deleterious for some samples.

**Delipidation and Elution of the SERCA1a-SLN Complex in the Presence of Detergent**—Solubilized material was recovered after centrifugation for 10 min at 50,000 \(\times g\) on a TLA100.3 rotor (Beckman coulter TL100 ultracentrifuge) and injected on a detergent equilibrated size exclusion column (TSK3000SW silica gel column, 50 mM MOPS-Tris at pH 7, 25 mM NaCl, and 0.4 mg/ml\(^{-1}\) DDM unless otherwise stated in the figure legends), essentially as previously described (26). Two 1-ml fractions were collected; one corresponding to the major peak known to contain SERCA1a (Ve\(_{0}\) ~6.5–7.5 ml), and a second fraction corresponding to the end of the previous elution peak (Ve\(_{0}\) ~8.0–9.0 ml), containing mainly mixed micelles and most of the SR endogenous lipids (27). Selected fractions were used for SDS-PAGE and MALDI-TOF analysis (see below and figure legends). For SDS-PAGE, two methods were used to visualize proteins after electrophoresis. The first technique consists of a classical Coomassie Blue staining (40% methanol (v/v), 10% acetic acid (v/v), and 0.1% Coomassie Blue R250 (w/v)). The second technique involves a light-induced modification of the tryptophanyl residues by haloalkane compounds (Mini-Protein TGX Stain-free Precast 4–20% gels from Bio-Rad) (28, 29). Whereas tryptophan has a fluorescence emission spectrum in the UV domain (about 310–350 nm, depending on its environment), the covalent indole derivative obtained after the reaction is now visible at a wavelength of about 410–420 nm. Assuming that the environment of each tryptophan in the SDS-containing gel is the same, the resulting in-gel fluorescence intensity can be used to estimate protein stoichiometries if the number of tryptophans in the sequences is known (29). Gels can be stained with Coomassie Blue in a subsequent step, following the above described procedure. Note that before assessing the stoichiometry of SERCA1a and SLN, which contain thirteen and only one tryptophan, respectively, this stain-free technique was validated on another complex purified in the laboratory.

**Analysis by MALDI-TOF Mass Spectrometry**—1 \(\mu l\) of sample was mixed with 3 \(\mu l\) of saturated solution of sinapinic acid in 30% acetonitrile, 0.3% trifluoroacetic acid. 1 \(\mu l\) of the mixture was loaded into a MALDI-TOF spectrometer (Perspective Biosystems, Voyager STR-DE) equipped with a nitrogen laser (337 nm). Spectra were obtained in linear mode using delayed mass spectrometry.

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extraction. Two ranges were analyzed: 2000–6000 Da for SLN alone (accelerating voltage of 20 kV) and 3000–140,000 Da for SLN and ATPase (accelerating voltage of 25 kV). The external standards used for calibration in the range 2000–6000 Da were *Escherichia coli* thioredoxin and insulin.

**Carbamidomethylation of Samples**—SEC-purified samples (2 μl at about 1 mg/ml−1), treated or not with NH₂OH were incubated with 50 mM iodoacetamide in 50 mM ammonium bicarbonate, for 15 min in the dark at room temperature. The reaction was stopped by addition of the matrix (sinapinic acid in 30% acetonitrile, 0.3% trifluoroacetic acid) and analyzed by MALDI-TOF as above.

**N-terminal Sequencing**—The following method was previously described (30): following SDS-PAGE separation of proteolytic peptides (performed on DOC-extracted SR vesicles) on Tricine gels (31) and transfer of the proteins onto a PVDF membrane, the bands corresponding to the various peptides were cut from the stained membranes and analyzed by Edman degradation. The sequence corresponding to the first 8 residues of SLN was found in the 6 kDa region.

**Steady-state ATPase Activity Measurements**—These measurements were performed using a classical enzyme-coupled assay, by measuring the rate of NADH oxidation (32, 33). The initial buffer for the assay was 50 mM Tes-NaOH pH 7.5, 1 mM MgCl₂, 100 mM KCl, 5 mM MgATP, 1 mM phosphoenolpyruvate, 0.1 mM l-malate, 0.05 mM pyruvate kinase, and 0.2–0.3 mM NADH (to reach about 1.5–1.7 OD at 340 nm). In some cases, 1 mM C₁₂E₈ or 2 mM 1-palmitoyl-sn-glycero-3-phosphorylcholine was used to stabilize the interactions among the proteins; this was performed under “Experimental Procedures.” DDM or C₁₂E₈ was used at 40 mg/ml−1.

**MALDI-TOF of hydrophobic peptides and large membrane proteins**—When required, endogenous lipids, use of low salt concentration and tight control of detergent concentration in the column were found to be the key points for obtaining mass spectrometry spectra by MALDI-TOF of hydrophobic peptides and large membrane proteins, such as SERCA1a. The spectrum obtained for the monomeric Ca²⁺-ATPase peak (Fig. 2A) revealed essentially the mass of intact Ca²⁺-ATPase, which, deduced from the amino acid sequence, is 109,532. The mass obtained by MALDI-TOF was 109,493.9 ± 93.1 Da (average of 10 independent experiments, taking into account the detection of the monoisonized species at about m/z ~109,500 together with its twice ionized equivalent at about m/z ~54,750). Two peptides of m/z ~4,011 and 4,036 were also present in the main SERCA1a peak (Fig. 2A, left inset). We postulated that these correspond to SLN (Mₙ = 3774 Da, mass of the protonated form (M + H)⁺), which interacts with the Ca²⁺-ATPase (see references in the introduction), but with some post-translational modifications, which would lead to their increased mass.

**RESULTS**

**Rabbit SLN Is Palmitoylated/Oleoylated on Cysteine 9**—MALDI-TOF mass determination of proteins in SR vesicles followed an established procedure (26). In short, SR vesicles were solubilized under different conditions and loaded on a detergent-containing SEC column (Fig. 1). Besides a small peak of aggregated SERCA1a in the void volume, solubilized Ca²⁺-ATPase elutes in a single peak as monomeric Ca²⁺-ATPase (25). A mixed micelle peak containing most of the lipids elutes after the main peak as smaller sized particles. Removal of endogenous lipids, use of low salt concentration and tight control of detergent concentration in the column were found to be the key points for obtaining mass spectrometry spectra by MALDI-TOF of hydrophobic peptides and large membrane proteins, such as SERCA1a. The spectrum obtained for the monomeric Ca²⁺-ATPase peak (Fig. 2A) revealed essentially the mass of intact Ca²⁺-ATPase, which, deduced from the yield of depalmitoylation and deoleoylation were very high.
reaching values of about 80% both after solubilization in DDM or C12E8 and low Mg2+/H11001 concentration. It was however less (about 30%) when hydroxylamine treatment was performed directly on the membrane or in DDM or C12E8 and high Mg2+/H11001 concentration (data not shown). No or very little unmodified SLN was detected in untreated samples (Fig. 2, panel A) or in a MALDI-TOF analysis of the detergent-lipid mixed micelles peak, which also contained SLN (not shown). This suggests that almost all the SLN present in the membrane, whether associated to SERCA1a or not, is modified, and that the fatty acids additions are not restricted to those in association with SERCA1a. The overall ratio between SLN and SERCA1a cannot be inferred from the peak sizes in MALDI-TOF experiments, but a rough estimate can be made from SDS-PAGE after modification of tryptophan by haloalkanes, or by Coomassie Blue staining (right inset of Fig. 2A). The comparison of the amount of SLN present before and after gel filtration indicates that a significant amount (about 70–80%) of the SLN is removed from the SERCA1a peak and is present in the mixed micelles peak (right inset of Fig. 2A). To further assess that cysteine 9 is the residue bearing the post-translational modification, we treated the proteins with iodoacetamide before or after hydroxylamine treatment (Fig. 3). Iodoacetamide only affected the peak corresponding to the deacylated SLN, with an expected increase of 57 Da corresponding to a carbamidomethylation of the free sulfhydryl group.

Crystallization Conditions and Sample Preparation Are Not Responsible for the Absence of Visible Palmitate/Oleate on the Recent SLN/SERCA1a Complex Structure—Because no cysteine modification was observed and modeled in the recent sarcolipin/SERCA1a crystal structures (1, 12) we wondered if the crystallization conditions could have promoted the loss of the fatty acids. Indeed palmitoylations are reversible reactions, and the enzymes necessary for palmitate addition or removal are
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FIGURE 3. Hydroxylamine and iodoacetamide treatment to unambiguously identify Cys-9 as the modified residue. Samples from the experiments depicted in Fig. 2, A and B were treated with iodoacetamide to unambiguously identify the palmitoylated/oleylated residues (panels A and B, respectively). As described above, 2 peaks were detected in the untreated sample (panel A; m/z = 4011.3 and 4036.0 Da). Treatment with hydroxylamine (+ NH₄OH, black arrow) resulted in the apparition of a unique peak at m/z = 3774.0 Da suggesting that post-translational modifications are present on a cysteine residue. Differences in masses of the peaks before and after treatment correspond to the mass of a palmitic acid and an oleic acid (cysteine residue). Differences in masses of the peaks before and after treatment correspond to the mass of a palmitic acid and an oleic acid (Δm/z = 236.2 and 262.8, respectively). Iodoacetamide was added on the two samples (untreated and hydroxylamine treated, + IAM, gray arrows). As depicted in panels C and D, iodoacetamide only affected the peak corresponding to the deacetylated SLN whose m/z increased from 3774.0 to 3830.5 Da: the difference should correspond to the acetyl group addition to the SH side-chain of the cysteine. IAN reaction was probably not total, as suggested by the presence of some residual unmodified SLN (*).

Pig SLN Is Also Palmitoylated/Oleoylated—Interestingly, many mammals have a phenylalanine instead of a cysteine in position 9 (see below for discussion on this subject). Considering the available sequences in protein sequence databases (UniProt KB and NCBI; see Fig. 7 legend for the detailed procedure), we chose pig as an alternative species bearing a cysteine to run similar tests. Pig SLN has the same sequence as the rabbit (right inset of Fig. 4). The SR preparation led to SDS-PAGE (Fig. 4A) and gel filtration profiles (not shown) that reveal a less pure preparation: a number of additional bands were present (compare pig or rabbit SR in Fig. 4A). Nevertheless, after gel filtration the SLN associated to the pig Ca²⁺-ATPase was clearly detected with the same two acylations, essentially also fully modified, with the noticeable feature that the oleoyl peak was larger than the palmitoyl peak in this case, representing about 60% of the peaks (Fig. 4B). As for the rabbit samples, hydroxylamine treatment led also to partial depalmitoylation/deoxygenation of pig SLN (Fig. 4C).

Deacylation Increases the Ca²⁺-ATPase Activity—Finally, we checked if the presence of palmitate/oleate affected the Ca²⁺-dependent ATPase activity of rabbit, pig, and rat SERCA1a, (the sarcolipin of the latter cannot be acylated as it contains a Phe instead of a Cys residue at position 9, see inset of Fig. 4). We found that NH₄OH treatment has a small effect on the activity of the detergent-solubilized enzyme, and on the activity of SR vesicles treated by the ionophore A23187 for the three mammals, whereas the affinity of SERCA1a for Ca²⁺ was unaltered (data not shown; tested only for the rabbit). In contrast, the Ca²⁺-ATPase activity was increased in the intact SR vesicles, by about 30% for the rabbit and 20% for the rat (Fig. 5, data not shown for pig SR, which, in a single experiment, was also increased).

DISCUSSION

There is an expanding list of mammalian proteins shown to be modified by S-palmitoylation (38), some of them being integral membrane proteins (39). Recent interesting additions in the context of skeletal muscle is a report describing palmitoylation of the rabbit ryanodine receptor RyR1, the α-subunit of the L-type calcium channel Cav1.1 and SERCA1a (40). For some species, we can now add SLN (see below). Furthermore, we report the presence of a rare modification, namely oleoylation in addition to palmitoylation of SLN. While oleoylation has been observed on the N-terminal methionine and on a lysine of the aquaporin AQP0 (41), this is to the best of our knowledge the first demonstration of oleoylation on a cysteine of a membrane protein. In addition, in the case of the AQP0, the two palmitoylated/oleoylated amino acid side chains are localized at the membrane interface. Surprisingly, in the case of SLN, the cysteine 9 side chain is about 8–10 Å below the membrane interface and neighboring Trp-931 from SERCA1a, as can be judged from the recent crystal structures, suggesting that the added acyl chain is diving deep into the membrane (Fig. 6A). This is also further demonstrated from a molecular dynamics simulation of SERCA1a in a lipid environment (42) as shown in Fig. 6B. At present we cannot provide any explanation for the present in the endoplasmic reticulum (for review, see Refs. 36, 37). Alternatively, the crystallization conditions may have favored the segregation of non-acylated SLN. Therefore, to approach the crystallization conditions in our experiments, we increased the Mg²⁺ concentration to 50 mM in the absence of Ca²⁺, during both the solubilization and the gel filtration steps, and we also changed the detergent DDM for C₁₂E₈. Apart from the formation of a larger amount of aggregates in the C₁₂E₈/50 mM Mg²⁺ condition, the MALDI-TOF results showed also full palmitoylation/oleoylation of the purified SLN (data not shown). This demonstrates that the fatty acids remain attached to SLN even in conditions close to the preparation of samples for crystallogenesis. We then wondered if the presence of fatty acid was linked to individual differences between rabbits used for the different SR preparation, due for instance to a difference in the feeding protocols or due to a slightly different preparation method of SR. The initial experiments (Figs. 1 to 3) were performed from rabbits raised in France. Using SR and DOC-extracted SR vesicles prepared in Denmark for the crystallization trials we obtained the same results as before, including about the same palmitate/oleate ratio (data not shown). Thus, we conclude that our initial observations were not linked to specific individuals or preparation procedures. Furthermore we conclude that SLN is fully palmitoylated/oleoylated prior to crystallization. We noted also that the Ca²⁺-ATPase was not very stable under such crystallization conditions while it is stable in the other conditions, even after hydroxylamine treatment (data not shown). The absence of the modification in the two recent crystal structures is likely the consequence of a large flexibility of the fatty chains in the crystal, preventing their observation in the crystal structure (see "Discussion").
A puzzling observation that about half of SLN carries a palmitate and the other half an oleate. One could assume that oleoylation could replace palmitoylation of a membrane protein with no particular functional consequences. Concerning the proposed extensive palmitoylation of SERCA1a reported by Chaube et al. (40), we cannot confirm it since the molecular mass that we obtained for this enzyme by MALDI-TOF (Fig. 2) corresponds exactly to that of the unmodified enzyme (except for the already described N-terminal acylation (43)). Furthermore, in previous works we identified peptides 1–119 or 1–181 (26, 44) and 351–
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FIGURE 6. Zoom-in on the sarcolipin Cys 9 side chain in the SERCA-SLN complex structure (PDB ID 4H1W). A, sarcolipin and SERCA1a are depicted with a green and a gray cartoon backbone, respectively, with the Cys-9 side chain in stick representation. The blue mesh represents the final refined 2Fo-Fc electron density map, contoured at 1.0 σ within a radius of 10 Å around SLN. The Cys-9 side chain is well defined in the density, but there is no indication for an attached acyl moiety. This does, however, not rule out the presence of a modification, since local structural disorder leads to a loss of diffraction signal, making highly mobile elements invisible in electron density maps. B, SERCA1a-SLN complex in a surface representation, colored as in panel A. Cys-9 (arrowhead) is highlighted in yellow. The position of the lipid bilayer is indicated by a ball-and-stick representation of a POPC bilayer (carbon, oxygen, nitrogen and phosphorus colored in gray, light red, light blue, and orange, respectively), derived from molecular dynamics simulations of SERCA in a lipid environment (42).

505 (45) as non-modified, which excludes the cysteine residues at position 12, 364 and 471 from the list given by (40). Also nearly full DTNB modifications of all 19 reduced cysteines of SERCA1a have been described in the past (46). Nevertheless, we cannot completely exclude a partial modification of one or two cysteines. Pig and rat SERCA1a appear also unmodified (data not shown). N-terminal sequencing experiments of rabbit SR in the low molecular mass region of Tricine-PAGE gels (about 6000–10,000 Da), revealed the presence of a peptide with the following sequence: MERSTREL, corresponding to MERSTREL, respectively, derived from molecular dynamics simulations of SERCA in a lipid environment (42).

4 M. le Maire and L. Denoroy, unpublished observation.
FIGURE 7. Alignment of available sarcolipin peptide sequences. The human SLN sequence (Uniprot no. O00631) was used as a template for a BLASTp search in the non-redundant protein sequence database (55). The PAM-30 algorithm was used as it is recommended for analysis of short peptide sequences. Following this strategy, 110 sequences were retrieved from the resulting taxonomy report, covering 67 species. However, when several sequences were available in the database for a given species (e.g., 13 sequences for human), all the available sequences were exactly the same, except for the cow and the ferret. In these two cases, a second SLN isoform was predicted from whole genome sequencing. Those sequences are identical in the membrane part but have putative additional residues in the N and/or C termini. The alignment presented here was done with the Seaview program (56, 57). Three sequences were shortened to optimize the figure presented here:

- **Bos taurus isoform X1**, residues M1SSLFASQSEKALPRAQKSRQPGRQEGVGAQVGEREEKPIAAQCVLDIPQDVRTSR-RSLVCPOQKAFSSHQATL75;
- **Anolis carolinensis**, residues R3KEARRCGWEAGFLPMPMOGNLVAE55;
- **Meleagris gallopavo**, residues L33FAKOTLEERWLST-FTGFCQHPFEV67.
surprising since the binding interface is state-specific (1) but not so surprising if one takes into consideration the crosslinking results of (7) showing that SLN can bind to various kinetic states of SERCA pump. We also noted that the palmitate/oleate ratio was not modified either under these various conditions.

What is then the role of palmitoylation/oleoylation? Charollais and van der Goot have reviewed a number of processes that are regulated by palmitoylation such as membrane protein folding, conformation of transmembrane domains (e.g. tilting of α-helices), trafficking, targeting, and association with specific membrane domains, the interplay with other post-translational modifications and interactions with other proteins (39, 48). Palmitoylation is reversible and the involved enzymes present in various membranes including the sarco/endoplasmic reticulum (36, 37). Thus, acylation/deacylation (when Cys-9 is present) is a regulatory modification of SLN, similar to SLN phosphorylation (3, 4) but this remains mainly an open question for the future. Phospholemman, a transmembrane peptide mem-

FIGURE 8. Cladogram representation of available SLN sequences. Sequences used as template for the cladogram construction are presented in Fig. 7. Sequence alignment and tree construction were done with the PhyML program starting from the 67 unique sequences (57). Prior to tree construction, sequence alignment was analyzed with the Gblock program to eliminate gaps and generate clearly defined flanking regions. The dendroscope program (58, 59) was used to enhance the visualization of the generated tree presented here. The color code is the following: amphibians in red, reptiles in green, fishes in brown, birds in orange, and mammals in blue. Cysteine-containing sequences are indicated with a red asterisk. Rabbit, pig, and rat SLN are bolded.
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