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Evolution

Calcium channels from *Cyprinus carpio* skeletal muscle

(voltage sensor/dihydropyridines)

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ABSTRACT **The complete amino acid sequence of the L-type calcium channel α_1 subunit from the carp (*Cyprinus carpio*) white skeletal muscle was deduced by cDNA cloning and sequence analysis. The open reading frame encodes 1852 amino acids (M_r 210,060). A 155-amino acid COOH-terminal sequence (after the fourth internal repeat) is evolutionarily preserved (90% homology) and may represent an important functional domain of L-type calcium channels. The photolabeled, membrane-bound, and purified carp α_1 subunits have masses of 211 and 190 kDa. The purified channel could not be phosphorylated by cAMP-dependent protein kinase. Two glycoproteins (α_2 subunits) are associated with the α_1 subunit and change their apparent masses from 235 and 220 kDa to 159 kDa upon reduction of disulfide bonds. Nucleic acid hybridization with α_2 cDNA revealed an 8.0-kilobase transcript in carp skeletal muscle. Evidence for a copurification of subunits similar in size to mammalian β or γ subunits was not obtained.**

Calcium antagonist receptors are abundant in mammalian skeletal muscle T-tubule membranes (1–3) and can be purified as a complex consisting of four subunits (α_1, α_2-δ, β, and γ) in an apparent 1:1:1:1 stoichiometry (4–7). Upon reconstitution in phospholipid vesicles or planar bilayers, the complex displays calcium channel activity that is enhanced by cAMP-dependent protein kinase (see, e.g., refs. 8–10). The functional roles of the α_2-δ subunit (11), the β subunit (12), and the γ subunit (13, 14) are not yet known. The α_1 subunit is homologous to the sodium channel α subunit, codes for a 212-kDa polypeptide (15), and carries the calcium antagonist binding domains as shown by photolabeling (7, 16). The α_1 subunit alone is sufficient to function as a voltage-dependent ionic pore when the full-length open reading frame of the cDNA is expressed in transfected murine L-cells (17). The importance of α_1 subunits for excitation–contraction coupling in skeletal muscle is emphasized by studies in mice with muscular dysgenesis. The calcium antagonist drug receptor (α_1 subunit) is absent in dysgenic skeletal muscle membranes and in myotubes grown in primary tissue culture (18). Injection of expression plasmids carrying skeletal muscle α_1 subunit cDNA into multinucleated dysgenic myotubes in primary tissue culture leads to the appearance of dihydropyridine-sensitive slow calcium currents (19) and restores excitation–contraction coupling even when cadmium blocks the ion flux (19). However, the α_1 subunit from rabbit cardiac muscle (20) could restore excitation–contraction coupling in dysgenic myotubes only in a fashion typical for cardiac muscle. Influx of calcium was required to activate the ryanodine-sensitive calcium release channel as shown by cadmium block or elimination of calcium from the extracellular medium (21). One would, therefore, expect that skeletal muscle α_1 subunits, in comparison to cardiac α_1 subunits, have some unique evolutionary preserved features that enable them to trigger the calcium release channel in the sarcoplasmic reticulum without conducting ions. Fish are the oldest and most diverse of the vertebrates and appear eminently suited to study molecular evolution (22). We have, therefore, undertaken the task to isolate and characterize components of the excitation–contraction coupling machinery from the carp *Cyprinus carpio*. We report the biochemical isolation of the calcium antagonist receptor from carp white skeletal muscle and the complete amino acid sequence of the carp α_1 subunit of the L-type calcium channel.§

EXPERIMENTAL PROCEDURES

Materials. Carp and rabbit skeletal muscle T-tubule membranes were purified as described (23). Saturation analysis of the isolated carp skeletal muscle microsomes with (+)-[^{3}H]PN 200-110 (at 25°C) yielded 9.5 ± 3.2 pmol of dihydropyridine sites per mg of protein with a K_d of 0.83 ± 0.27 nM (n = 5). Purification of carp skeletal muscle calcium antagonist receptors was similar to the procedure described for mammalian skeletal muscle (24), with the following modifications: 200–300 mg of microsomal protein was prelabeled at 37°C for 30 min with 6–7 nM (+)-[^{3}H]PN 200-110 in 50 mM Tris·HCl, pH 7.4/0.1 mM phenylmethylsulfonyl fluoride. After centrifugation at 4°C and 47,000 × g (10 min), the pellet was resuspended at 4°C in 1% digitonin/50 mM Tris·HCl/500 mM NaCl containing protease inhibitors (24). After 20 min of stirring at 4°C and centrifugation at 47,000 × g for 60 min, the supernatant was forced through a 0.2-μm (pore size) nitrocellulose filter and purified by wheat germ agglutinin chromatography and sucrose gradient centrifugation as described (24). Combined peak fractions from the sucrose gradients were applied onto a 3-ml heparin-Sepharose column equilibrated with 0.1% digitonin/50 mM Tris·HCl, pH 7.4/10 mM NaCl containing protease inhibitors. After sample application, the column was washed with 50 ml of the buffer above followed by a linear gradient of 10–500 mM NaCl. (+)-[^{3}H]PN 200-110-prelabeled receptors were eluted between 230 and 270 mM NaCl. Synthetic calcium channel peptides were prepared as described (25) and coupled with glutaraldehyde to bovine serum albumin at a molar ratio of 6:1 (26). Antibody F4/4 was raised against peptide P4 (EERKRRKLSRGLPDK, amino acid residues 679–693 from the rabbit skeletal muscle α_1 subunit, except that Met-686 was replaced by Leu) in New Zealand White rabbits and immunoreactivity was tested by ELISA. Before use, F4/4 was successively preadsorbed by affinity columns (Affi-Gel 10, Bio-Rad), to which bovine serum albumin, carp serum proteins, and the break-through fraction (devoid of dihydropyridine receptors) of the wheat germ agglutinin col-

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M37203).

umn step were coupled, according to the procedures given by the manufacturer.

Photoaffinity Labeling and Phosphorylation. Photoaffinity labeling of particulate calcium channels with (−)-[^{3}H]azidopine (45 Ci/mmol; 1 Ci = 37 GBq) and the arylazide phenylalkylamine [N-*methyl*-^{3}H]LU 49888 (78 Ci/mmol) was performed as described (27). To study the effects of solubilization and purification, 6.4 nM (−)-[^{3}H]azidopine was incubated in the dark with skeletal muscle microsomal protein at 1 mg/ml (rabbit) or 1.3 mg/ml (carp) at 25°C for 45 min, centrifuged 15 min at 47,000 × *g*, and resuspended in 4 ml of 50 mM Tris·HCl, pH 7.4/0.1 mM phenylmethylsulfonyl fluoride. After 20 min of UV-irradiation (TL 36, Philips-Dulphar, Amsterdam) on ice at a distance of 10 cm, samples were centrifuged as above and the pellets were solubilized and photolabeled calcium channels were purified by wheat germ agglutinin-Sepharose chromatography. Freeze-dried samples were analyzed by SDS/gel electrophoresis and fluorography (27). Nonspecific photoincorporation was followed as above with 1 μM (±)-PN 200-110. Phosphorylation studies with cAMP-dependent protein kinase were performed exactly as described by Hymel *et al.* (9).

Immunoblot Analysis, Glycoprotein Staining, and Deglycosylation. Purified calcium channel complexes from rabbit skeletal muscle (sucrose gradient and wheat germ agglutinin column step) or from the carp (sucrose gradient step) were heated in electrophoresis sample buffer (27) with 10 mM dithiothreitol at 56°C and loaded onto preparative 8% gels containing SDS. After electrophoresis, proteins were transferred to membranes [Immobilon-P, poly(vinylidene difluoride); Millipore] by using a SemiPhor semidry transfer unit (Hoefer). Membranes were blocked in Tris-buffered saline (TBS; 50 mM Tris·HCl, pH 7.4/0.15 M NaCl) containing 0.2% gelatine, 1% nonfat dry milk, 0.1% Tween 20, and 0.5% Triton X-100 (blocking buffer). Membranes were cut into 3-mm strips and incubated with antisera in blocking buffer containing 2% bovine serum albumin for 12 hr at 4°C. Each incubation step was followed by three 10-min washes with TBS containing 0.5% Triton X-100 and 0.1% Tween 20. To evaluate the specificity of the antisera, synthetic peptides (25 μg/ml) were added. Immunoreactivity was detected by incubation with anti-rabbit IgG-alkaline phosphatase conjugate for 1.5 hr and developing in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. Glycoproteins were stained with concanavalin A as described (28). Deglycosylation was performed with recombinant N-Glycanase (Genzyme) according to the manufacturer's protocol.

RNA Blot Hybridizations. Total RNA was isolated from lateral–caudal white muscle of carp according to the guanidinium thiocyanate procedure (29) followed by ultracentrifugation using a 5.7 M CsCl cushion. Poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose affinity chromatography (30), electrophoresed on 1% agarose gels containing 6% (vol/vol) formaldehyde (31), and transferred to Nytran membranes (Schleicher & Schuell) according to the manufacturer's protocol. Northern blots were hybridized in 5× SSPE (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4)/5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/0.7% SDS/50% (vol/vol) formamide/calf thymus DNA (150 μg/ml) at 43°C for 15 hr. Blots were probed with the α_1 subunit rabbit skeletal muscle clone pSkmCaCha1.7 (11) [base pairs (bp) 1006–5314] and with a rabbit skeletal muscle α_2 subunit full-length clone (11) after labeling with [α-^{32}P]dCTP by using a random-priming kit (Pharmacia) according to ref. 32. Filters were washed under low-stringency conditions in 2× SSC (1× SSC = 0.15 M NaCl/0.01 M sodium citrate, pH 7.0)/0.1% SDS at 43°C.

Cloning Strategy and Sequence Analysis. Poly(A)$^+$ RNA (3 μg) was transcribed to cDNA after oligo(dT) priming by using a Pharmacia cDNA synthesis kit. *Eco*RI-adaptor-linked cDNAs were ligated into λgt10 vector and packaged using Gigapack (Stratagene) packaging extracts, giving a library of 6 × 10^7 plaque-forming units/μg of mRNA on *Escherichia coli* C600 hfl. Duplicate filters from master library plates were screened using the oligolabeled clone pSkmCaCh1.7 (11), which yielded 3 positive clones: K5 (bp 420–2625), K6 (bp 3364–4097), and K3 (bp 3944–6022). Rescreening was carried out with adequate fragments of these clones to yield 12 additional clones covering the entire coding region for the carp α_1 subunit. The occurrence of positive clones seen in this library was 1 in 2 × 10^5. cDNA inserts were cleaved in appropriate parts with rare restriction enzymes and subcloned into M13mp18 and M13mp19 vectors, and overlapping clones were sequenced on both strands by the dideoxynucleotide chain-termination method (33, 34) using adenosine 5′-[α-[^{35}S]thio]triphosphate (Amersham). All sequence analyses and comparisons were carried out using LKB 2020 DNASIS/PROSIS software (Hitachi, San Bruno, CA).

RESULTS

Properties of Carp Skeletal Muscle Calcium Antagonist Receptors. Photoaffinity labeling experiments of carp microsomal membranes and quantitation of specifically incorporated radioactivity {(−)-[^{3}H]azidopine} by slicing SDS gels yielded sizes of 211 ± 7 kDa and 190 ± 9 kDa for the carp α_1 subunit. [N-*methyl*-^{3}H]LU 49888 specifically photolabeled the entire 170- to 211-kDa region in carp microsomes and the region of 170 ± 12 kDa in rabbit skeletal muscle α_1 subunit (data not shown). After solubilization and a three-step purification, the dihydropyridine receptors were enriched to 1191 ± 321 pmol/mg of protein (n = 4). The polypeptide pattern of the purified carp skeletal calcium antagonist receptor was clearly distinct from that of the guinea pig or the rabbit. Two glycoproteins had apparent sizes of 235 ± 5 kDa and 220 ± 3.9 kDa under alkylating conditions. These glycoproteins copurified with (+)-[^{3}H]PN 200-110 binding activity (Fig. 1) and migrated as a diffuse band of 159 ± 4.1 kDa upon disulfide bond reduction. In analogy to the mammalian skeletal muscle α_2 subunit, we term these glycoproteins "α_2 subunits." Deglycosylation drastically changed the silver-stained polypeptide pattern, yielding three new polypeptides of 183, 171, and 165 kDa under alkylating conditions by SDS/PAGE. Upon reduction of the deglycosylated preparation, two new polypeptides of 107 and 101 kDa were observed. A polypeptide of 111 ± 7 kDa was consistently observed in the carp calcium antagonist receptor complex but only under reducing conditions. This polypeptide did not stain with concanavalin A and was also present in fractions that were devoid of (+)-[^{3}H]PN 200-110 binding activity (data not shown). For 16 purifications of the carp skeletal muscle calcium antagonist receptors, we could not resolve the α_1 subunit as a well-defined silver- or Coomassie brilliant blue-stained band upon SDS/gel electrophoresis although faintly stained polypeptides in the 190- to 210-kDa region were often observed (see Fig. 1). [N-*methyl*-^{3}H]LU 49888 specifically photolabeled the entire 170- to 211-kDa region but could not resolve individual polypeptides (data not shown). These results suggested heterogeneity and evidence for this was obtained by immunoblot analysis as well as by solubilization and partial purification of (−)-[^{3}H]azidopine-photolabeled carp skeletal muscle microsomes. Specifically photoincorporated radioactivity was found in 211- and 190-kDa polypeptides (Fig. 1). Immunoblot analysis with the antibody F4/4 identified two prominent bands of 211 and 190 kDa and two faint bands of 170 and 225 kDa in the carp preparation. For the purified rabbit skeletal muscle, we observed a prominent 164-kDa polypeptide and faint polypeptides of 179 and 203 kDa. Immunostaining was specific in all cases because peptide P4 completely suppressed immunoreactivity (Fig. 1). No newly phosphorylated

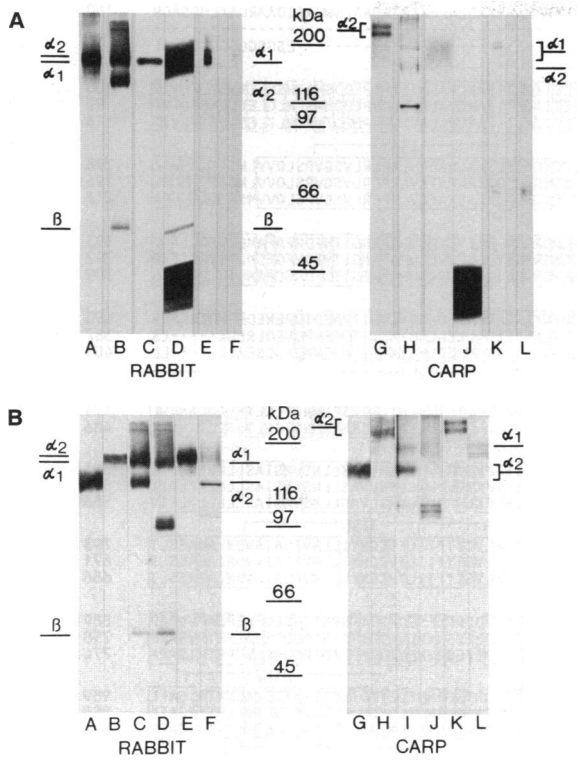

FIG. 1. (*A*) Polypeptide composition, photoaffinity labeling, phosphorylation, and immunoblot analysis of rabbit and carp skeletal muscle calcium channels. Proteins were analyzed in 8% gels containing SDS either with reduction of disulfide bonds (10 mM dithiothreitol; lanes B–F and H–L) or after alkylation (10 mM *N*-ethylmaleimide; lanes A and G). Lanes A–F contain purified rabbit skeletal muscle calcium channels. Lanes A and B contain 1.4 μg of protein, silver stained. Lane C contains 10 μg of protein, photolabeled with (−)-[^{3}H]azidopine (27) and fluorogram exposed for 5 weeks. Lane D shows the phosphorylation pattern (3 μg of protein); autoradiograms were exposed for 96 hr. The intensely phosphorylated polypeptide in the region below 45 kDa represents the catalytic subunit of the cAMP-dependent protein kinase. Lane E shows an immunoblot with 1.5 μg of protein; preadsorbed antibody F4/4 was used at final dilution of 1:1000. Lane F is as in lane E but with peptide P4 present. Lanes G–L contain carp skeletal muscle calcium channels, analyzed as above. Lane G and H contain 1.5 μg of protein from a three-step purified preparation, stained with silver. Lane I contains (−)-[^{3}H]azidopine-photoaffinity-labeled carp skeletal muscle microsomes (70 μg of protein, partially purified by wheat germ agglutinin-Sepharose); the fluorogram was exposed for 5 weeks. Photoincorporation was specific as 1 μM (±)-PN200-110 completely suppressed photolabeling (data not shown). Lane J shows the phosphorylation pattern (4 μg of purified channel protein). Lane K shows an immunoblot with 5.3 μg of wheat germ agglutinin-Sepharose-purified receptor protein; preadsorbed antibody F4/4 was used as in lane E. Lane L is as in lane F. (*B*) Glycosylation of calcium channel subunits. Conditions for SDS/gel electrophoresis were as in *A* either with reduction of disulfide bonds (lanes A, C, D, G, I, and J) or after alkylation (lanes B, E, F, H, K, and L). Lanes A–F contain rabbit skeletal muscle calcium channels and lanes G–L contain carp skeletal calcium channels. Lanes: A and B, 4 μg of purified channel protein, stained with concanavalin A; C and E, 5 μg of purified channel protein, silver-stained; D and F, 5 μg of purified channel protein, digested for 5 hr with recombinant N-Glycanase, silver stained; G and H, 3 μg of two-step purified carp calcium antagonist receptor protein, stained with concanavalin A; I and K, 4 μg of protein, silver stained; J and L, 4 μg of protein, digested for 5 hr with recombinant N-Glycanase.

polypeptides were observed in the α_1 subunit region when the purified carp calcium antagonist receptors were treated with the catalytic subunit of the cAMP-dependent protein kinase, whereas the α_1 and β subunits from the rabbit are phosphorylated under the same conditions (Fig. 1).

Cloning and Sequence Analysis of the α_1 Subunit from the Carp Skeletal Muscle. The size of the α_1 gene product was estimated at 6.5 kilobases (kb) by Northern blot hybridization analysis (Fig. 2), which is the same as for the rabbit α_1 subunit (11, 20). Fig. 2 also shows that the α_2 transcripts in carp (8.0 kb) and mammalian tissue (7.5 kb) (11) are of similar size. The nucleotide sequence of the α_1 subunit was determined from 6103 bp in both orientations. An open reading frame encodes 1852 amino acids with a calculated molecular weight of 210,060 for the core protein (Fig. 3). The nucleotide sequence surrounding the first ATG codon after the last in-frame nonsense codon TGA of the 5′ untranslated region agrees reasonably well with the favored sequence flanking an initiation codon (35). A consensus polyadenylylation signal (AATAAA) (36) was found 268–273 bp after the stop codon terminating the reading frame. Nevertheless, no poly(dA) tract could be detected in the following 88 nucleotides downstream. Hydropathy plots (37) and secondary structure prediction studies (38) suggest that the carp skeletal muscle α_1 subunit has the same transmembrane topology as the rabbit skeletal and heart muscle α_1 subunits. According to this model five potential N-glycosylation sites (39) are predicted to be located on the extracellular loops (Asn-99, -102, -275, -471, and -1158). Asn-275 is common to the three α_1 subunits. Four potential cAMP-dependent protein kinase phosphorylation sites (40, 41) are found on the putative cytoplasmic side (Thr-407 and Ser-1471, -1523, and -1738). Only one, Ser-1523, is common to the three α_1 subunits. Calculating only identical amino acids, the degree of overall sequence similarity aligning the three α_1 subunits is 46%. The overall homology between two equivalent organs (skeletal muscle) of the two evolutionarily distant species (rabbit and carp) is 65%, which is higher than the 56% similarity between the two organs (skeletal muscle and heart) of the rabbit. The degree of similarity differs widely in various regions of the proteins. Sequences of the highest and of the lowest homologies can be found in the putative cytoplasmic hydrophilic COOH terminus. By comparing the three aligned sequences of the COOH-terminal region, the first 155 residues immediately after the last transmembrane segment IVS6 (Fig. 3) show a high sequence similarity of 90%, followed by a region of 75 residues with a moderate 36% identity. Within a region of 409 amino acids (including a large insertion for the rabbit heart α_1 subunit) without more than random sequence similarities (except some few small blocks), a region of 32% homology extending over 38 residues exists. Putative intracellular loops connecting transmembrane segments show similarity values of 67.9 ± 19.2%, which is comparable with the overall homology of intramembrane segments (73.3 ± 14.1%) and is higher than that of extracellular loops (42.6 ± 16.1%).

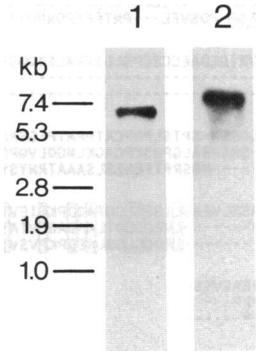

FIG. 2. Northern blot analysis of carp white skeletal muscle poly(A)$^+$ RNA (4 μg) probed with the α_1 rabbit skeletal muscle cDNA clone pSkmCaCha1.7 (11) (lane 1) and with a full-length α_2 cDNA clone (11) (lane 2).

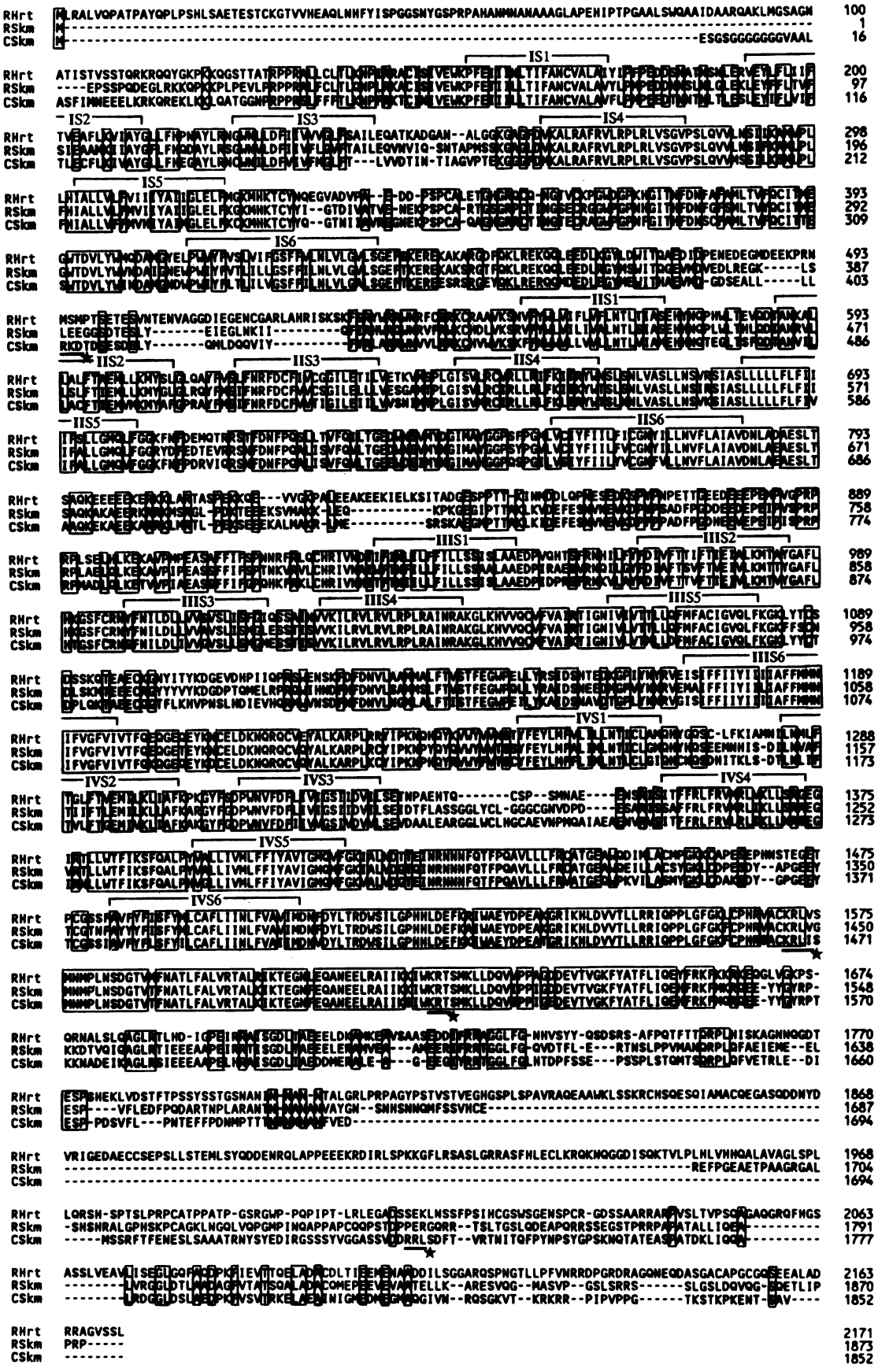

FIG. 3. Comparison of the deduced amino acid sequences of three α_1 subunits. The sequence derived from rabbit heart (RHrt) (20) is aligned with that of rabbit skeletal muscle (RSkm) (15) and compared to carp skeletal muscle (CSkm). The numbers of the amino acid residues are given on the right, starting with the first methionine after the initiation codon. The putative transmembrane segments S1–S6 in each of the repeats I–IV are indicated above the lines. Only identical residues conserved in both organs as well as in both species are boxed. Asterisks and bars indicate potential phosphorylation sites for cAMP-dependent protein kinase.

DISCUSSION

Despite an evolutionary distance of ≈350 million years, the carp skeletal muscle α_1 subunit shows some striking invariant regions when compared to the mammalian L-type calcium channel α_1 subunits. Most notable is the highly conserved putative intracellular region encompassing 155 amino acids immediately after the fourth internal repeat. We suggest that this region contains functional domains that are typical or essential for all L-type calcium channels, regardless of whether they couple to ryanodine receptors, conduct ions, or both. On the other hand a comparison of the carp and rabbit skeletal muscle α_1 subunits with the cardiac α_1 subunit indicates that domains exist that are unique for skeletal muscle. This invites consideration of their functional significance to regulate the calcium release channel in the sarcoplasmic reticulum. Ser-687 of the rabbit skeletal muscle α_1 subunit is rapidly phosphorylated *in vitro* by cAMP-dependent protein kinase (42). This site is missing in the carp α_1 subunit. Other potential phosphorylation sites (Thr-407 between internal repeats I and II and Ser-1471, Ser-1523, and Ser-1738 in the COOH-terminal region) exist in the carp α_1 subunit but we were unable to label the purified complex with [γ-^{32}P]ATP and the catalytic subunit of the cAMP-dependent protein kinase, as was also reported for the chicken cardiac calcium channel (43). The photolabeled α_1 subunits in carp skeletal muscle microsomes were of greater apparent size when electrophoresed next to the rabbit or guinea pig preparation in SDS gels. Partial purification of photoaffinity-labeled carp microsomes did not yield a single species of radioactive α_1 subunits and immunoblot analysis revealed size heterogeneity of the purified polypeptides. Possible explanations are proteolytic cleavage (44) or heterogeneity of the carp white muscle, where three types can be discriminated on the basis of histochemical differences (45). The glycosylated α_2-δ subunit, which is associated with L-type calcium channels in various tissues (46), exhibits a shift in mobility on SDS gels upon disulfide-bond reduction similar to that of the two copurifying carp glycoproteins. This behavior suggests that the carp glycoproteins may be related to the mammalian α_2-δ subunit. We obtained evidence by nucleic acid hybridization that an α_2 transcript is present in carp skeletal muscle. Evidence for a copurification of subunits in the size range of mammalian β (52 kDa) or γ (30–33 kDa) subunits was not obtained. Thus the subunit composition and the size of the receptor of the calcium channel complex from the carp skeletal muscle differ from their mammalian counterparts. It will be of interest to investigate if the carp skeletal muscle α_1 subunit can restore excitation–contraction coupling in dysgenic myotubes or can be functionally expressed as a slow calcium channel, as was shown for its mammalian counterpart (47).

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