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Structural and functional correlates of a mutation in the malignant hyperthermia-susceptible pig ryanodine receptor

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The skeletal muscle ryanodine receptor of malignant hyperthermia-susceptible (MHS) pigs contains a mutation at residue 615 that is highly correlated with various abnormalities in the regulation of sarcoplasmic reticulum (SR) Ca^{2+} channel activity. In isolated SR membranes the Arg^{615} to Cys^{615} ryanodine receptor mutation is now shown to be directly responsible for an altered tryptic peptide map, due to the elimination of the Arg^{615} cleavage site. Furthermore, trypsin treatment released 86–99 kDa ryanodine receptor fragments encompassing residue 615 from the SR membranes. We conclude that the 86–99 kDa domain containing residue 615 is near the cytoplasmic surface of the ryanodine receptor and likely near important Ca^{2+} channel regulatory sites.

Sarcoplasmic reticulum; Ryanodine receptor; Malignant hyperthermia; Mutation; Calcium release channel

1. INTRODUCTION

The skeletal muscle sarcoplasmic reticulum (SR) ryanodine receptor/Ca^{2+} channel is the pathway by which stored Ca^{2+} is released into the sarcoplasm during excitation–contraction coupling [1]. This homotetrameric complex of 565 kDa subunits, seen as a quatrefoil structure in electron micrographs [1], has been sequenced from the cDNA [2]. Many reports of alterations in the activity of the porcine skeletal muscle ryanodine receptor (e.g. [3–6]) have made it the most likely candidate gene for conferring susceptibility to the metabolic disorder malignant hyperthermia (MH). In support of this hypothesis Fujii et al. [7] have recently identified a nucleotide base mutation (resulting in the replacement of Arg^{615} with Cys^{615}) in the ryanodine receptor cDNA from MH-susceptible (MHS) pigs. Thus, this natural mutation may be utilized to gain further insight into the structure and function of a channel critical to the regulation of muscle contraction. In this report we have identified 86 and 99 kDa tryptic fragments of the normal and MHS pig ryanodine receptor that contain residue 615. Our results indicate that this domain is near both the surface of the protein and important channel regulatory sites.

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2. MATERIALS AND METHODS

Pietrain pigs homozygous for the MH gene [8], Yorkshire and Minnesota no. 1 pigs homozygous for the normal gene [8,9], and Near-Pietrain pigs derived from more than 4 generations of backcrossing halothane-negative pigs to Pietrains [9], were obtained from the closed herd maintained at the University of Minnesota Experimental Farm. MH susceptibility was determined by exposure to 3% halothane (in 97% oxygen) by mask, with a positive test response indicated by limb muscle rigidity in less than 4 min.

Trypsin treatment of heavy SR membranes from MHS and normal pigs was at 37°C for 10 min in a medium containing 1 M sucrose, 20 mM Tris-HCl, pH 7.0, 1 mg/SR protein/ml, and the indicated trypsin concentration [10]. SR proteins were electrophoresed on 3–12% gradient polyacrylamide gels and analyzed on immunoblots with sheep anti-ryanodine receptor antisera and horseradish peroxidase conjugated rabbit anti-sheep IgG secondary antibodies [10]. Sheep antibodies specific for the MHS and normal ryanodine receptor tryptic fragments were affinity-purified off of Immobilon strips [11] containing the indicated ryanodine receptor tryptic fragments.

Anti-ryanodine receptor antibody beads were prepared by coupling sheep antibodies (affinity-purified from a rabbit ryanodine receptor column) to CNBr-activated Sepharose 4B. For immunoprecipitation of the 86 kDa ryanodine receptor fragment, trypsin treated SR membranes were solubilized in the presence of 1 M NaCl, 2% CHAPS, 1% phosphatidylcholine, 50 mM Tris-HCl, pH 7.4, and a protease inhibitor cocktail. The soluble extract was then added to an aliquot of anti-ryanodine receptor immunobeads and incubated at 4°C for 4 h. Specifically bound ryanodine receptor fragments were eluted from the immunobeads by addition of electrophoresis buffer containing 5% 2-mercaptoethanol and 2% SDS. The eluted protein was fractionated on polyacrylamide gels and the N-terminal sequence obtained from transfers onto Immobilon membranes [12].

Conditions for the polymerase chain reaction amplification of a 74 bp ryanodine receptor sequence from porcine genomic DNA were as described by Fujii et al. [7]. Sequence of the PCR product was determined by the dideoxy chain termination method [13], following blunt-end ligation into the *Sma*I site of pBluescript. Dot blot hybridization

of ^{32}P-labeled oligonucleotide probes to the PCR products was also as described by Fujii et al. [7], with 5'-TGGCCGTGCGCTCCAAC-3' for the C1843 allele and 5'-GGCCGTGTGCTCCAA-3' for the T1843 allele.

3. RESULTS

The immunoblot shown in Fig. 1A indicates the presence of the high M_r ryanodine receptor in the porcine heavy SR preparations prior to trypsin treatment. In addition to the intact ryanodine receptor two lower M_r components were also observed (Fig. 1A), likely due to a partial proteolysis of this protein during SR membrane preparation. Fig. 1B (lanes 1–4) shows an immunoblot of trypsin-treated SR membranes, where ryanodine receptor doublet fragments of M_r approximately 99 kDa were detected in MHS SR, while normal SR contained ryanodine receptor doublet fragments of both 99 and 86 kDa. The figure also shows that the use of higher trypsin concentrations resulted in the loss of these components (lanes 8–12). Following centrifugation of trypsin-digested MHS and normal SR for 30 min at 150,000 × g, the 99 and 86 kDa ryanodine receptor fragments were detected in the resulting supernatant (Fig. 1C, lanes 5–8) and absent from the pellet (lanes 9–12). The 99 and 86 kDa ryanodine receptor fragments could not be differentiated from the numerous other SR protein fragments on Coomassie blue-stained gels (not shown). When purified ryanodine receptor preparations (isolated by the method of Lai et al. [1]) were treated with trypsin we were unable to detect the 86 and 99 kDa fragments on immunoblots, although numerous other fragments were detected (not shown).

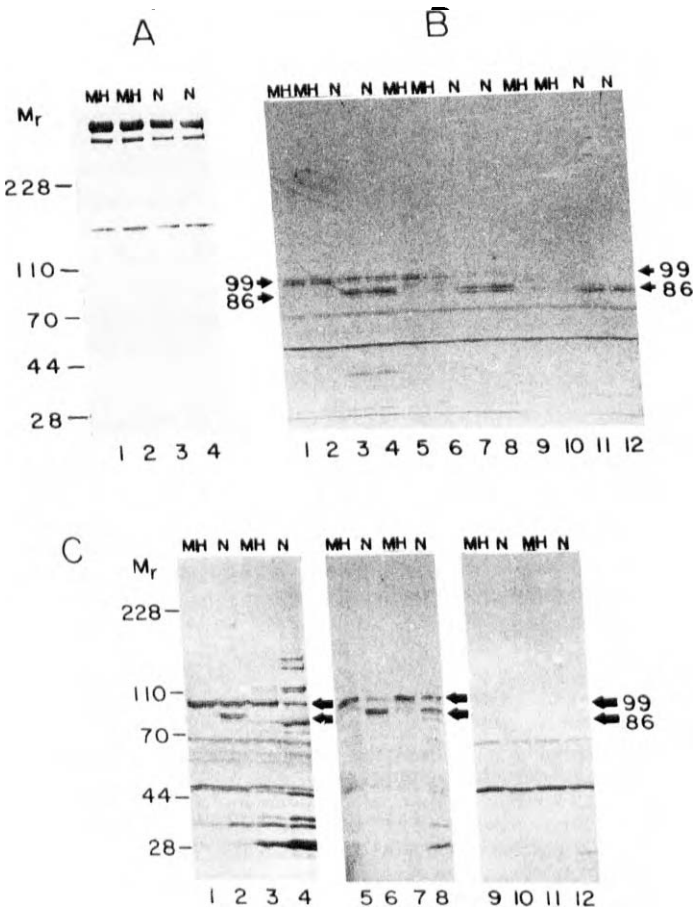

Fig. 1. Tryptic immunopeptide maps of the MHS and normal SR ryanodine receptor. Heavy SR vesicles from 2 different MHS and 2 different normal pigs were incubated in the absence of trypsin (A) or in the presence of various trypsin concentrations (B) for 10 min at 37°C. Panel B: lanes 1–4, trypsin to SR weight ratio of 1:320; lanes 5–8, trypsin to SR ratio of 1:240; lanes 9–12, trypsin to SR ratio of 1:160. Panel C: 1 MHS and 1 normal SR preparation were treated with trypsin (1:240), after which they were diluted with an equal volume of 0.3 M sucrose and centrifuged at 150,000 × g for 30 min. Samples of trypsin-treated SR (lanes 1–4), and the resultant supernatant (lanes 5–8) and pellets (lanes 9–12) were analyzed. Lanes 1,2,5,6,9,10 — 10 min trypsin incubation; lanes 3,4,7,8,11,12 — 5 min trypsin incubation. All samples were analyzed for ryanodine receptor immunoreactive fragments as described in section 2. The positions of molecular weight markers are indicated on the left, and the positions of the 99 and 86 kDa ryanodine receptor fragments are indicated by the single arrowheads.

Affinity-purified antibodies to the 99 and 86 kDa ryanodine receptor fragments were prepared and utilized to stain immunoblots. Fig. 2C shows that antibodies to the normal 99 kDa tryptic fragments recognized the 86 kDa normal fragments, as well as the 99 kDa MHS fragments. Similarly, antibodies to the 86 kDa normal fragments recognized both the 99 kDa normal and the 99 kDa MHS fragments (Fig. 2D), and antibodies to the 99 kDa MHS fragments recognized both the 99 and 86 kDa normal fragments (Fig. 2E). Thus, the 86 and 99 kDa tryptic fragments appear to arise from the same domain of the ryanodine receptor.

Antibody beads were used to immunoprecipitate the 86 kDa normal ryanodine receptor fragment from trypsin-treated normal SR. The major immunoprecipitated component detected on Coomassie blue-stained gels had a M_r of 86 kDa, and was recognized on immunoblots by the anti-ryanodine receptor antibody (data not shown). The N-terminal sequence of this fragment indicated significant homology to residues 617–627 of the rabbit ryanodine receptor (Fig. 3A), just downstream from a potential trypsin cleavage site at Arg^{615}. DNA corresponding to this region of the porcine ryanodine receptor gene was then amplified by the polymerase chain reaction and sequenced (Fig. 3B). The nucleotide sequences between the PCR primers were identical to those determined by Fujii et al. [7], with a C in position 1843 of the normal Yorskhire DNA and a T in position 1843 of the MHS Pietrain DNA, translating into the Arg^{615} to Cys^{615} mutation.

Dot blots of the 74 bp porcine DNA PCR product were hybridized to either the C1843 (normal) or T1843 (MHS) ryanodine receptor oligonucleotide probes (Fig. 4). This confirmed that all pigs from the MHS Pietrain herd utilized for our previous studies of ryanodine receptor alterations [5,6,9,10], contained only the T1843 allele, while the normal Yorkshire and Minnesota no.

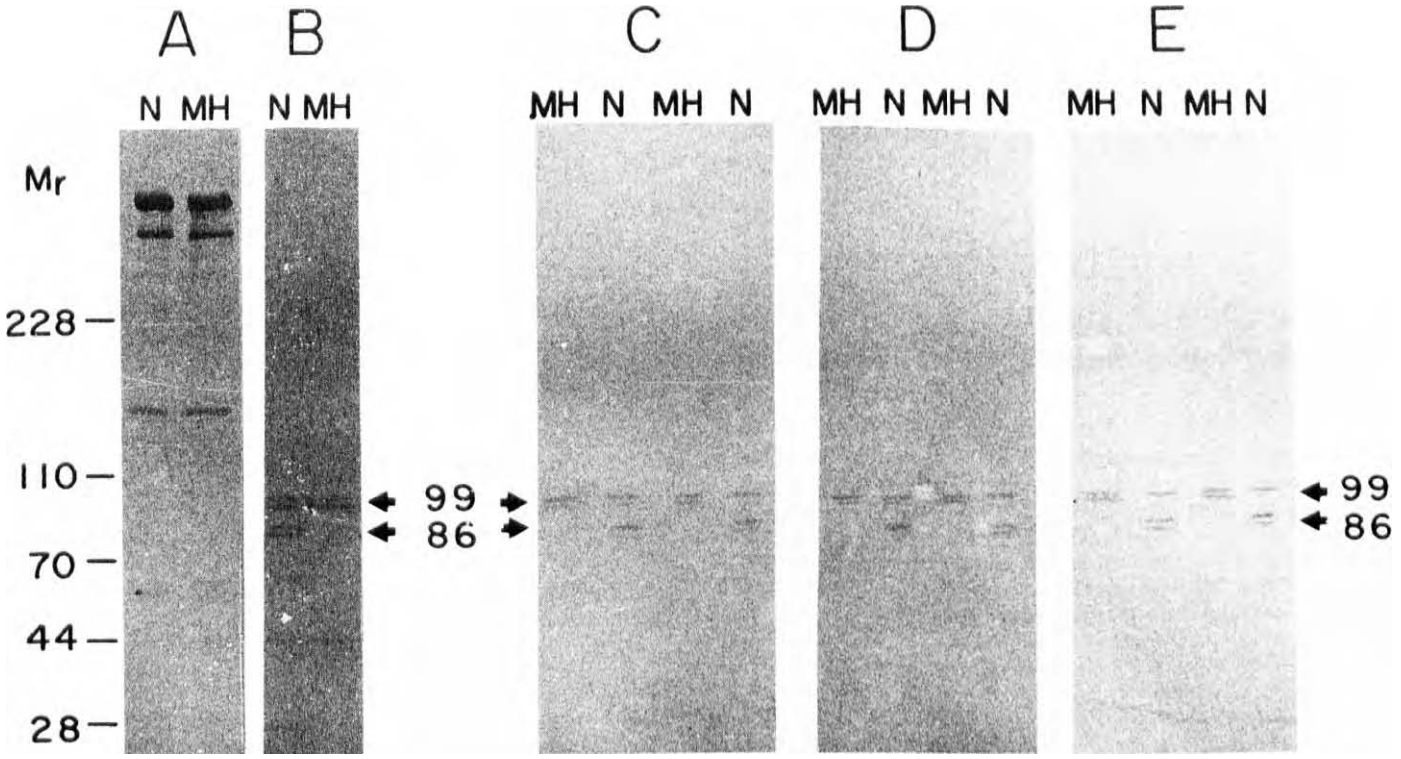

Fig. 2. Immunoblot analysis of the 99 and 86 kDa ryanodine receptor tryptic fragments. MHS and normal heavy SR membranes were incubated in the absence (A) or presence of trypsin at a 1:320 weight ratio for 10 min (B–E). Antibodies utilized to stain the transfers were either the sheep anti-ryanodine receptor antiserum (A and B), or sheep antibodies that had been affinity purified from immunoblots of the ryanodine receptor tryptic fragments: (C) anti-normal 99 kDa antibodies; (D), anti-normal 86 kDa antibodies; (E), anti-MHS 99 kDa antibodies.

l herds contained the C1843 allele (Fig. 4A). We also examined families of pigs in which the oligonucleotide hybridization results could be correlated with the results of the halothane-challenge test for MH susceptibility

(A)

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                              615                   620                   625
Rabbit                        Arg Ser Asn Gln Asp Leu Ile Thr Glu Asn Leu Leu Pro

Pig 86 kD Fragment              X  Asn Gln Asp  X  Ile  X  Glu Asn Leu Leu Pro
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(B)

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                                  1843
1828 AAT GGT G                      ⇓                          ATT ACT GAG 1869

Normal          TG GCC GTG CGC TCC AAC CAA GAT CTC
                Val Ala Val Arg Ser Asn Gln Asp Leu

MHS             TG GCC GTG TGC TCC AAC CAA GAT CTC
                Val Ala Val Cys Ser Asn Gln Asp Leu

                                  615                   620
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Fig. 3. N-Terminal sequence of the 86 kDa ryanodine receptor tryptic fragment and DNA sequence of the ryanodine receptor PCR product. (A) Normal SR was incubated with trypsin (1:160 for 10 min at 37°C), the 86 kDa normal ryanodine receptor fragment was immunoprecipitated, and the N-terminal sequence was determined from an Immobilon transfer as described in section 2. Amino acid residues which could not be determined are represented with an X. Sequence and numbering scheme of the rabbit skeletal muscle ryanodine receptor [2] is provided. (B) MHS Pietrain and normal Yorkshire genomic DNA were utilized for the production of the 74 bp ryanodine receptor DNA segment as described in section 2. These PCR products were ligated into pBluescript and sequenced. Partial sequence of the PCR primers (covered with solid lines) and the ryanodine receptor cDNA sequence nucleotide numbering system determined by Fujii et al. [7] are provided.

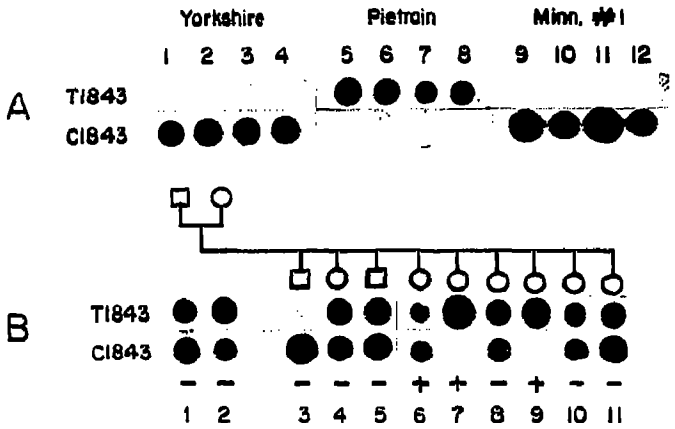

Fig. 4. Dot blot oligonucleotide hybridization analysis of the 74 bp ryanodine receptor PCR product. (A) Four different MHS Pietrain, normal Yorkshire, and normal Minnesota no. 1 pigs were analyzed. (B) Near-Pietrain parents and their progeny. The figures represent the autoradiogram that resulted from hybridization to the ^{32}P-labeled C1843 and T1843 oligonucleotides as described in section 2. The response of each pig to the halothane-challenge test for MH is also given, with + indicating a positive halothane response and − indicating a negative halothane response.

(Fig. 4B). In this example the halothane-negative Near-Pietrain parents had copies of each ryanodine receptor allele (i.e. were heterozygotes) and the progeny of this mating demonstrated all three possible genotypes. Interestingly, one heterozygote pig (individual no. 6) reacted positively in the halothane-challenge test. That all homozygotes for the T1843 allele were MHS (in agreement with Fujii et al. [7]), while heterozygotes based on the oligonucleotide hybridization assay could be either positive or negative in the halothane-challenge test (Fig. 4B), has been a consistent finding in the 8 Near Pietrain pedigrees examined to date. This appears to indicate ambiguities associated with applying the in vivo challenge test for the identification of heterozygotes.

4. DISCUSSION

This study confirms our previous observation of the generation of 99 kDa MHS and 86 kDa normal porcine SR ryanodine receptor tryptic fragments [10], and demonstrates that a normal 99 kDa ryanodine receptor fragment can also be detected (Fig. 1B). Affinity- purified antibodies to each of these fragments recognized the other two (Fig. 2), indicating that these three fragments were all derived from the same domain of the ryanodine receptor. The 86 kDa normal ryanodine receptor tryptic fragment had residue 616 as its N terminus (Fig. 3A) and the DNA sequences of the MHS and normal ryanodine receptors in this region confirmed the Arg^{615} to Cys^{615} mutation described by Fujii et al. [7] (Fig. 3B). These data thus indicate that the Arg^{615} to Cys mutation in the MHS pig ryanodine receptor eliminates an accessible trypsin cleavage site at residue 615, such that the normal 86 kDa fragment cannot be formed. That these 99 and 86 kDa fragments were not observed when purified MHS and normal ryanodine receptors were subjected to proteolysis may indicate an altered conformation of this molecule in the detergent-solubilized purified state.

Our results suggest that Arg^{615} may be on the surface of the native ryanodine receptor molecule, as it is accessible to trypsin. The 86–99 kDa tryptic fragments are released from the SR (Fig. 1C), indicating that they are derived from the cytoplasmic foot region [1,2]. Arg^{615} may also be located near important regulatory sites, as the MHS and normal porcine ryanodine receptors demonstrate differences in Ca^{2+} regulation of Ca^{2+} release [3–5], channel gating [6], and [^{3}H]ryanodine binding activity [5,9]. To date none of the various ligand binding sites (e.g. Ca^{2+}, Mg^{2+}, ATP, calmodulin [1]) which regulate this channel have been identified. Further study of the MHS porcine ryanodine receptor mutation should help define structure/function relationships within this Ca^{2+} channel protein.

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