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Specific Absence of the α_1 Subunit of the Dihydropyridine Receptor in Mice with Muscular Dysgenesis*

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Muscular dysgenesis is a lethal mutation in mice that results in a complete absence of skeletal muscle contraction due to the failure of depolarization of the transverse tubular membrane to trigger calcium release from the sarcoplasmic reticulum. In order to determine whether the defect in muscular dysgenesis leads to a specific loss of one of the components of excitation-contraction coupling or to a generalized loss of all components of excitation-contraction coupling, we have analyzed skeletal muscle from control and dysgenic mice for the sarcoplasmic reticulum and transverse tubular proteins which are believed to function in excitation-contraction coupling. We report that the proteins involved in sarcoplasmic reticulum calcium transport, storage, and release ((Ca^{2+} + Mg^{2+})-ATPase, calsequestrin, and calcium release channel) are present in dysgenic muscle. Also present in dysgenic muscle is the 175/150-kDa glycoprotein subunit (α_2) of the dihydropyridine receptor. However, the 170-kDa dihydropyridine binding subunit (α_1) of the dihydropyridine receptor is absent in dysgenic muscle. These results suggest that the specific absence of the α_1 subunit of the dihydropyridine receptor is responsible for the defects in muscular dysgenesis and that the α_1 subunit of the dihydropyridine receptor is essential for excitation-contraction coupling in skeletal muscle.

The molecular mechanisms involved in coupling transverse tubular membrane depolarization to sarcoplasmic reticulum calcium release are not understood. Recently the protein responsible for releasing calcium from the junctional sarcoplasmic reticulum, the calcium release channel, has been identified and purified from rabbit skeletal muscle (1–4). The calcium release channel is identical to the junctional "foot" protein in skeletal muscle (2) and has been shown to consist of a single polypeptide of approximately 450,000 Da (1). The dihydropyridine receptor of the transverse tubular membrane has recently been proposed to be a voltage sensor for skeletal muscle excitation-contraction coupling (5, 6) and thus may be involved in the activation of the calcium release channel. The dihydropyridine receptor has been purified from rabbit skeletal muscle and shown to consist of two higher molecular weight protein subunits and at least two lower molecular weight subunits (7). The high molecular weight subunits include the 170-kDa dihydropyridine binding subunit named α_1 and the 175/150-kDa glycoprotein subunit named α_2, which shifts from 175 to 150 kDa upon reduction (7).

Muscular dysgenesis (mdg) (8, 9) is a lethal autosomal recessive mutation in mice that results in the complete lack of excitation-contraction coupling in all skeletal muscle from these mice (10). Dysgenic muscle resembles normal muscle with respect to the ability of the sarcolemma to generate action potentials (10), the sarcoplasmic reticulum to release calcium in response to caffeine, and the contractile proteins to respond to calcium (11). Dysgenic muscle differs from normal muscle in that depolarization fails to induce the release of calcium from the sarcoplasmic reticulum. Consistent with this functional defect, electron microscopy of embryonic dysgenic skeletal muscle has shown that triads, the membranous region at which excitation-contraction coupling occurs, are reduced in number and abnormal in morphology (12). Attempts to further characterize the defect in dysgenic muscle has shown that the dihydropyridine-sensitive calcium current is missing in dysgenic myotubes (13) and that there is a reduction in high affinity dihydropyridine binding (14) in dysgenic skeletal muscle homogenates. Thus, the *mdg* mutation clearly and specifically prevents excitation-contraction coupling and provides an excellent model with which to study and identify the molecular components of excitation-contraction coupling.

EXPERIMENTAL PROCEDURES

Isolation of Normal and Dysgenic Skeletal Muscle Membranes—Adult mouse skeletal muscle and newborn normal or dysgenic skeletal muscle were dissected, frozen in liquid nitrogen, and stored at −70 °C. The samples were then homogenized using a Brinkmann Polytron PTA-7 in 10 volumes of buffer A containing sodium pyrophosphate (20 mM), sodium phosphate monobasic (20 mM), $MgCl_2$ (1 mM), EDTA (0.5 mM), sucrose (303 mM), and the following protease inhibitors: aprotinin (76.8 nM), benzamidine (0.83 mM), iodoacetamide (1 mM), leupeptin (1.1 μM), pepstatin A (0.7 μM), and PMSF[1] (0.2 mM). Homogenates were centrifuged for 10 min in a TJ-6R Beckman centrifuge at 1500 × *g*. Supernatants were removed, and pellets were homogenized in buffer A and centrifuged two additional times as described above. The three supernatants were combined and centrifuged at 150,000 × *g* in a Beckman Type 50.2 Ti rotor. Membranes were resuspended in a minimum volume of buffer A. Adult rabbit microsomes were isolated by a modification of the method of Mitchell *et al.* (15, 16). Protein was determined by the method of Lowry *et al.* (17) as modified by Peterson (18).

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[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacryamide gel electrophoresis.

Culturing and Solubilization of Myotubes—Primary cultures of myoblasts were prepared from fore- and hindlimbs of newborn normal and dysgenic mice as previously described (13, 19). Briefly, limb muscle is minced, digested with collagenase, filtered, and cells are then plated onto 35-mm Falcon "primaria" dishes or 25-cm^2 primaria flasks (Falcon). Myotubes were allowed to form and mature (10–12 days). Cultures were washed with calcium- and magnesium-free Ringer's solution (155 mM NaCl, 5 mM KCl, and 10 mM HEPES, pH 7.4 with NaOH), scraped from the dishes into calcium- and magnesium-free Ringer's solution containing 10 μM aprotinin, pelleted, and rapidly frozen for storage at −70 °C.

Myotubes were thawed, weighed, and quickly solubilized in 20 volumes (v/w) of solubilization buffer (6% SDS, 50 mM dithiothreitol, 10 mM EDTA, 100 μg/ml benzamidine, 40 μg/ml PMSF, 185 μg/ml iodoacetamide, 0.5 M sucrose, and 130 mM Tris, pH 6.8, with HCl). Samples were vortexed, heated for 5 min in boiling water, and centrifuged to remove any remaining particulate matter. Protein was quantitated by the method of Lowry *et al.* (17) as modified by Peterson (18) after precipitation with 5% trichloracetic acid in the presence of 0.5 mg of sodium deoxycholate.

SDS-PAGE and Immunoblot Analysis—Skeletal muscle membranes or isolated myotubes were analyzed by SDS-PAGE (3–12% gradient gels) using the buffer system of Laemmli (20) and stained with Coomassie Blue or transferred to nitrocellulose according to Towbin *et al.* (21). Nitrocellulose blots were stained with various antibodies as described (22–25) using secondary antibodies conjugated to either horseradish peroxidase or alkaline phosphatase.

Monoclonal antibodies against calsequestrin, the (Ca^{2+} + Mg^{2+})-ATPase, and the 170-kDa dihydropyridine binding subunit of the dihydropyridine receptor have been previously prepared (23–25). Polyclonal antibodies directed against the calcium release channel were previously prepared (25) by injecting guinea pigs with purified receptor according to the method of Tung (26). Polyclonal antibodies against the α_2 subunit were prepared using SDS gel slices according to the method of Tung (26) as previously described (25).

Materials—Electrophoretic reagents were obtained from Bio-Rad and molecular weight standards from Bethesda Research Laboratories. Protease inhibitors were from Sigma. Peroxidase and alkaline phosphatase-conjugated secondary antibodies were obtained from Boehringer Mannheim and Cappel. All other chemicals were of reagent grade quality.

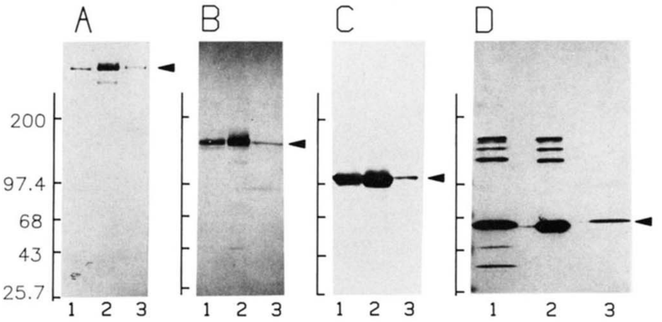

FIG. 1. **Immunoblots of adult mouse, adult rabbit, and neonatal mouse skeletal muscle.** Each *panel* contains adult mouse membranes (*lane 1*), adult rabbit membranes (*lane 2*), and newborn mouse membranes (*lane 3*) stained with antibodies against the calcium release channel (*panel A*), the α_1 subunit (*panel B*), the (Ca^{2+} + Mg^{2+})-ATPase (*panel C*), and calsequestrin (*panel D*). *A*, blots are stained with polyclonal guinea pig anti-calcium release channel antibodies followed by incubation with rabbit anti-guinea pig IgG conjugated to alkaline phosphatase. Protein loaded: *lane 1,* 20 μg; *lane 2,* 20 μg; *lane 3,* 75 μg. *B*, blots are stained with monoclonal mouse antibody IIID5 directed against the α_1 subunit of the dihydropyridine receptor followed by incubation with goat anti-mouse IgG conjugated to horseradish peroxidase. Protein loaded: *lane 1,* 50 μg; *lane 2,* 25 μg; *lane 3,* 200 μg. *C*, blots are stained with monoclonal mouse antibody VE12 against the (Ca^{2+} + Mg^{2+})-ATPase followed by incubation with goat anti-mouse IgG conjugated to horseradish peroxidase. Protein loaded: 50 μg/lane. *D*, blots are stained with monoclonal mouse antibodies VIIID1$_2$, VIIA7, VIH1, and ID1$_c$ against calsequestrin (63 kDa) followed by incubation with goat anti-mouse IgG conjugated to horseradish peroxidase. Protein loaded: *lane 1,* 80 μg; *lane 2,* 30 μg; *lane 3,* 150 μg. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left (prestained molecular weight standards were from Bethesda Research Laboratories and are as follows: myosin, 200,000 Da; phosphorylase *b*, 97,400 Da; bovine serum albumin, 68,000 Da; ovalbumin, 43,000 Da; and α-chymotrypsinogen, 25,700 Da).

RESULTS AND DISCUSSION

To determine the defect in muscular dysgenesis we have examined normal and dysgenic muscle for the protein constituents of the sarcoplasmic reticulum and transverse tubular membrane involved in excitation-contraction coupling. Since the quantity of neonatal dysgenic muscle is limited (approximately 0.1 g/mouse), we have used indirect immunoperoxidase staining of protein blots (Western or immunoblots) from normal and dysgenic muscle for this analysis. Initially, we tested monoclonal and polyclonal antibodies previously prepared in our lab against adult rabbit proteins for cross-reactivity with proteins of similar molecular weight in newborn and adult mouse muscle. Fig. 1 shows that the calcium release channel, (Ca^{2+} + Mg^{2+})-ATPase, calsequestrin, and the α_1 subunit of the dihydropyridine receptor are present in newborn and adult mouse muscle and are recognized by various antibodies. The α_2 subunit of the dihydropyridine receptor was also recognized by a polyclonal antibody against rabbit α_2, but antibodies to the rabbit β and γ subunits did not cross-react with normal mouse membranes.

In Fig. 2, membranes were prepared from newborn normal (+/*mdg*?) mice and dysgenic (*mdg*/*mdg*) mice and were tested for the presence of the sarcoplasmic reticulum calcium release channel, the (Ca^{2+} + Mg^{2+})-ATPase, and calsequestrin. Each of these proteins is present, but in reduced amounts, in dysgenic muscle. The two proteins specific to the triad junction, the sarcoplasmic reticulum calcium release channel and calsequestrin, are diminished more than the (Ca^{2+} + Mg^{2+})-ATPase. The reduction in the calcium release channel (junctional foot protein) and in calsequestrin (electron dense content of the terminal cisternae) is consistent with the electron microscopic data showing a reduced number and an abnormal morphology of triad structures in dysgenic muscle (12, 14). The presence of calsequestrin and the (Ca^{2+} + Mg^{2+})-ATPase is consistent with results (27) which show that the mutation does not affect the synthesis of these two proteins in dysgenic myotubes.

The effect of the *mdg* mutation on the α_1 and α_2 subunits of the dihydropyridine receptor was also examined. Fig. 3 shows that the 175/150-kDa glycoprotein (α_2 subunit) is detected at near normal levels in dysgenic membranes, while the 170-kDa protein (α_1 subunit) of the dihydropyridine receptor is not detected. Immunoblots with both polyclonal and monoclonal antibodies against the α_1 subunit indicate that the α_1 subunit is absent in dysgenic muscle. The mutation does not appear to result in an altered or truncated α_1 subunit, since bands of altered size were not detected in dysgenic muscle. To determine whether very low levels of the α_1 subunit might be present in dysgenic muscle, several immunoblots were reacted multiple times with anti-α_1 subunit antibodies. Under these conditions, a faint band of approximately 170 kDa was detected in dysgenic muscle. However, these conditions also produced nonspecific labeling in both control and dysgenic muscle samples. The 170-kDa band obtained following multiple staining may represent a substantially decreased level of the α_1 subunit in dysgenic skeletal myofibers or may represent a protein originating from vascular tissue. To differentiate between these alternatives primary cultures of normal and dysgenic muscle myotubes were analyzed for the presence or absence of the same sarcoplasmic reticulum and transverse tubular proteins as discussed above. Analysis of myotubes eliminates the possibility of postmortem degrada-

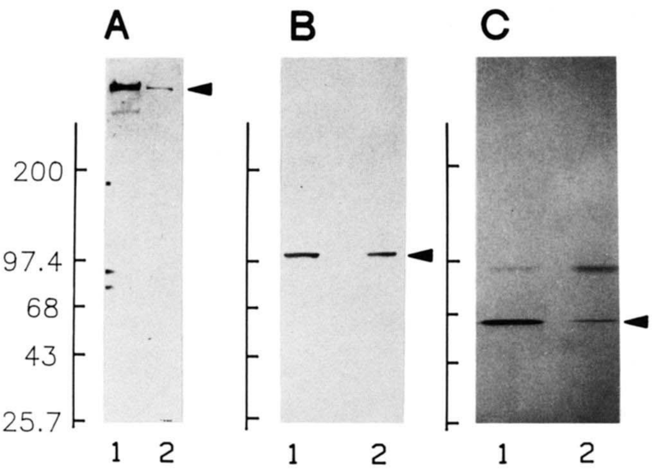

FIG. 2. **Immunoblots of newborn normal (+/*mdg?*) and dysgenic (*mdg/mdg*) mouse skeletal muscle membranes.** Each *panel* contains normal membranes (*lane 1*) and dysgenic membranes (*lane 2*) stained with antibodies against the calcium release channel (*panel A*), the (Ca^{2+} + Mg^{2+})-ATPase (*panel B*), and calsequestrin (*panel C*). *A*, blots are stained with polyclonal guinea pig anti-calcium release channel antibodies followed by incubation with rabbit anti-guinea pig IgG conjugated to horseradish peroxidase (100 μg of protein/lane). *B*, blots are stained with monoclonal mouse antibody VE12 against (Ca^{2+} + Mg^{2+})-ATPase followed by incubation with goat anti-mouse IgG conjugated to horseradish peroxidase (50 μg of protein/lane). *C*, immunoblots are stained with monoclonal mouse antibodies VIIID1$_2$, VIIA7, VIH1, and ID1$_c$ against calsequestrin (63 kDa) followed by incubation with goat anti-mouse IgG conjugated to horseradish peroxidase (150 μg of protein/lane). The band near 95 kDa represents a nonspecific reaction of the secondary goat anti-mouse antibody with the newborn mouse membranes (data not shown). The identity of this band is not known. Molecular weights ($M_r \times 10^{-3}$) are indicated on the *left*.

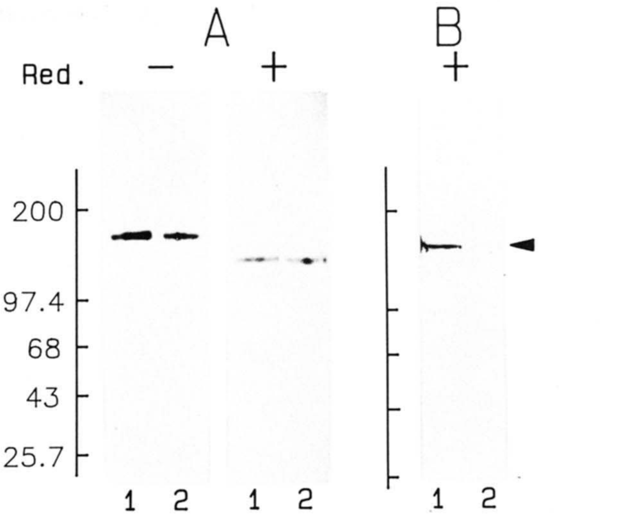

FIG. 3. **Immunoblots of normal (+/*mdg?*) *and dysgenic* (*mdg/mdg*) neonatal mouse skeletal muscle membranes.** Each *panel* contains normal membranes (*lane 1*) and dysgenic membranes (*lane 2*) stained with antibodies against the α_2 subunit (*panel A*) and the α_1 subunit (*panel B*) of the rabbit skeletal muscle dihydropyridine receptor. *A*, blots are stained with polyclonal guinea pig antibodies against the α_2 subunit followed by incubation with rabbit anti-guinea pig IgG conjugated to horseradish peroxidase. All lanes contain 250 μg of protein. Protein samples were electrophoresed under nonreducing conditions containing 20 mM *N*-ethylmaleimide (−) or under reducing conditions containing 10 mM dithiothreitol (+) for 10 min at room temperature. *B*, blots are stained with polyclonal antibody against the α_1 subunit followed by incubation with goat anti-guinea pig IgG conjugated to horseradish peroxidase (100 μg of protein/lane). Molecular weights ($M_r \times 10^{-3}$) are indicated on the *left*.

tion that may occur in newborn dysgenic mice and of potential vascular tissue contamination which may be present in membranes prepared from limb muscle. Fig. 4 shows that the sarcoplasmic reticulum calcium release channel and the (Ca^{2+} + Mg^{2+})-ATPase are detected in near normal levels in dysgenic myotubes. Even with multiple staining, the 170-kDa protein (α_1 subunit) of the dihydropyridine receptor could not be detected in dysgenic myotubes. These results further illustrate the specificity of the defect in muscular dysgenesis for the α_1 subunit of the dihydropyridine receptor.

The results described here show that despite the overwhelming loss of one critical component of triads (α_1 subunit of the dihydropyridine receptor), expression of other genes crucial for excitation-contraction coupling is only moderately affected. Thus, we propose that all other abnormalities reported in dysgenic muscle can be attributed to the selective loss of the α_1 subunit. These include the absence of the dihydropyridine-sensitive calcium current (13), reduced dihydropyridine binding (14), failure of excitation-contraction coupling (10), and abnormal morphology of the triad junction (14). Our results provide strong evidence that the α_1 subunit of the dihydropyridine receptor is involved in coupling transverse tubular depolarization to sarcoplasmic reticulum calcium release and support the hypothesis that the dihydropyridine receptor acts as a voltage sensor in the transverse tubular membrane for excitation-contraction coupling.

The muscular dysgenic mutation could lead to depletion of the α_1 subunit by several different routes. First, the mutation might alter a regulatory region of the gene for the α_1 subunit and thereby decrease transcription. Second, the mutation may alter the structural gene such that the gene produces a nonfunctional, and therefore labile, product. Third, the primary

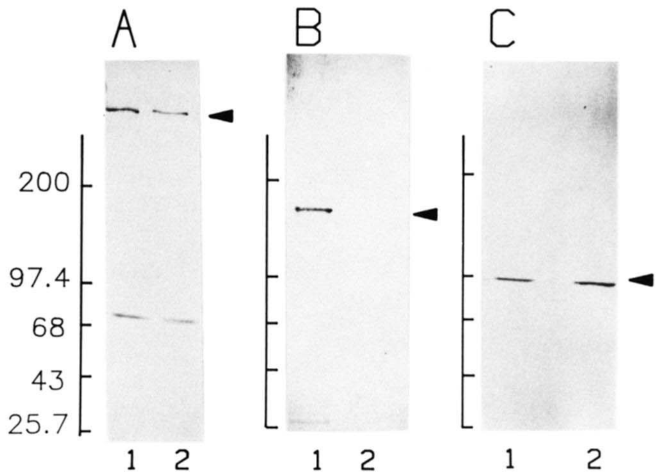

FIG. 4. **Immunoblots of normal (+/*mdg?*) and dysgenic (*mdg/mdg*) myotubes grown in primary culture and solubilized in SDS.** Each *panel* contains normal myotubes (*lane 1*) and dysgenic myotubes (*lane 2*) stained with antibodies against the calcium release channel (*panel A*), the α_1 subunit (*panel B*), and (Ca^{2+} + Mg^{2+})-ATPase (*panel C*). *A*, blots are stained with polyclonal guinea pig anti-calcium release channel antibodies followed by incubation with rabbit anti-guinea pig IgG conjugated to horseradish peroxidase (100 μg of protein/lane). *Arrowhead* denotes position of calcium release channel. *B*, blots are stained with monoclonal mouse antibody IIID5 against the α_1 subunit of the dihydropyridine receptor followed by incubation with goat anti-mouse IgG conjugated to horseradish peroxidase (200 μg of protein/lane. *C*, blots are stained with monoclonal mouse antibodies VE12 and VIE8 against the (Ca^{2+} + Mg^{2+})-ATPase followed by incubation with goat anti-mouse IgG conjugated to horseradish peroxidase (150 μg of protein/lane). Molecular weights ($M_r \times 10^{-3}$) are indicated on the *left*.

defect may prevent the production, by nonmuscle cells, of a trophic signal necessary for normal muscle development. The third hypothesis is supported by results which show that coculturing myotubes with spinal cord cells restores contractility (28) and calcium currents (29) to a fraction of the myotubes. On this basis Rieger *et al.* (29) suggest that the mutation prevents motor neurons from suppling a factor which activates a gene required for excitation-contraction coupling in skeletal muscle. The recent demonstration that normal fibroblasts are able spontaneously to fuse with myotubes in culture and bring about functional rescue of dysgenic muscle (30) provides an alternative explanation to the proposal of Rieger *et al.* (29). Specifically fibroblasts or other non-neuronal cells in the normal spinal cord preparation could fuse with dysgenic myotubes and thus provide the necessary genetic information for proper calcium currents and excitation contraction-coupling. In fact, we feel it is unlikely that the absence of a trophic factor would produce a complete loss of only the α_1 subunit as we have shown here.

The observation that the sarcoplasmic reticulum calcium release channel is substantially expressed in dysgenic muscle is consistent with caffeine-induced contractures previously demonstrated in dysgenic muscle (11). The near normal levels of the calcium release channel in dysgenic myotubes as compared to the greatly reduced level in *mdg* muscle suggest that the absence of the α_1 subunit of the dihydropyridine receptor in *mdg* muscle results in reduced expression of the calcium release channel. Preliminary results suggest that the dihydropyridine receptor may be in close association with the sarcoplasmic reticulum calcium release channel (junctional foot protein) (31, 32), and thus the absence of the α_1 subunit may prevent proper formation of the triadic junction in *mdg* muscle.

Schneider and Chandler (33) originally proposed that charged groups in the membrane of the transverse tubular system were displaced by changes in voltage and that this displacement could directly regulate the release of calcium from the sarcoplasmic reticulum. Rios and Brum (5) have proposed that the dihydropyridine receptor is the voltage sensor in the transverse tubular membrane originally described by Schneider and Chandler. The lack of spontaneous contraction (in spite of spontaneous action potentials) in dysgenic myotubes suggests that the absence of the α_1 subunit prevents the normal activation of the calcium release channel. Additionally, the relaxed state of dysgenic myotubes suggests that the calcium release channel is closed even in the absence of the α_1 subunit of the dihydropyridine receptor in dysgenic myotubes. Thus, the α_1 subunit of the dihydropyridine receptor cannot act as a gate or "plunger" in the release of calcium from the sarcoplasmic reticulum (33) but instead may regulate calcium release via some other less direct mechanism. Thus, despite the recent identification of two of the molecular components which are required for normal excitation-contraction coupling, the calcium release channel and the α_1 subunit of the dihydropyridine receptor, the mechanism by which these proteins interact to induce sarcoplasmic reticulum calcium release remains unknown.

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