

## Phosphorylation of $\alpha$ -syntrophin is responsible for its subcellular localization and interaction with dystrophin in muscle cells

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Received 20 September 2019; revised 07 November 2019

Syntrophin is a well-known adaptor protein that links intracellular proteins with the dystrophin-glycoprotein complex (DGC) at the sarcolemma. However, little is known about the underlying mechanism that regulates the intracellular localization of  $\alpha$ -syntrophin and its interaction with dystrophin. In this study, we demonstrate that  $\alpha$ -syntrophin phosphorylation determines its intracellular localization and interaction with dystrophin in muscle cells.  $\alpha$ -Syntrophin, a predominant isoform in skeletal muscles, directly interacts with ion channels, enzymes, receptors, and DGC proteins. Despite  $\alpha$ -syntrophin being a potential signaling molecule, most studies focus on its function as a dystrophin-associated protein. However, we previously reported that  $\alpha$ -syntrophin has a variety of DGC-independent functions to modulate cell migration, differentiation, survival, and protein stability. According to the results of the *in vitro* phosphorylation assays using subcellular fractions, the phosphorylated  $\alpha$ -syntrophin accumulated only at the plasma membrane, and this event occurred regardless of dystrophin expression. However, the  $\alpha$ -syntrophin interacting with dystrophin at the membrane was not in a phosphorylated state. We also identified that protein kinase C (PKC) was involved in the phosphorylation of  $\alpha$ -syntrophin, which restricted  $\alpha$ -syntrophin to interact with dystrophin. In conclusion, we demonstrate that the phosphorylation of  $\alpha$ -syntrophin by PKC regulates its intracellular localization and interaction with dystrophin.

**Keywords:** Intracellular localization, Immunoprecipitants, Protein kinase C, Protein-protein interaction

Syntrophins are peripheral membrane proteins first identified in Torpedo postsynaptic membranes<sup>1</sup>. Syntrophin family is composed of  $\alpha$ ,  $\beta$ 1,  $\beta$ 2,  $\gamma$ 1, and  $\gamma$ 2 isoforms.  $\alpha$ -Syntrophin is predominant isoform in cardiac and skeletal muscles, whereas  $\gamma$ 1- and  $\gamma$ 2-syntrophins are mainly expressed in brain tissue<sup>1,2</sup>. In skeletal muscles, syntrophins are a component of the dystrophin-glycoprotein complex (DGC) located at the sarcolemma<sup>3,4</sup>. In the absence of dystrophin, syntrophins fail to localize at the sarcolemma<sup>5</sup>.  $\alpha$ -Syntrophin directly interacts with dystrophin *via* its syntrophin unique (SU) domain<sup>6,7</sup>, however, calmodulin inhibits this interaction in a  $\text{Ca}^{2+}$ -dependent manner<sup>8</sup>. In addition, syntrophins also bind to diacylglycerol kinase- $\zeta$  (DGK- $\zeta$ ) in skeletal muscles and the complex of syntrophin-DGK- $\zeta$  is translocated from the cytosol to the plasma membrane<sup>9</sup>.

The intracellular localization of proteins is closely related to their functions<sup>10</sup>. The post-translational modification such as phosphorylation can modulate

the localization of proteins by regulating the protein-protein interaction, signal transduction, and protein stability<sup>11-14</sup>. Both dystrophin and syntrophin can be phosphorylated *in vivo* by various kinases<sup>15,16</sup>. The  $\text{Ca}^{2+}$ -calmodulin-dependent protein kinase II (CaM kinase II) phosphorylates both dystrophin and syntrophin<sup>17,18</sup>. Interestingly, the interaction of these two proteins is inhibited by CaM kinase II phosphorylation<sup>18</sup>. These reports suggest that the interaction between dystrophin and syntrophin can be regulated by the phosphorylation status of these two proteins. Syntrophin has primarily been considered as an adaptor protein that links intracellular signaling molecules to DGC by interacting with various proteins such as aquaporin-4 (AQP-4) and nitric oxide synthase (nNOS)<sup>2,18</sup>.

During myoblast differentiation,  $\alpha$ -syntrophin is expressed from the early differentiation stages at which point dystrophin is not yet expressed<sup>19</sup>. We have previously found novel functions of  $\alpha$ -syntrophin in myoblast differentiation<sup>20</sup> and migration<sup>21</sup>, which are independent of dystrophin. In this study, we focused on  $\alpha$ -syntrophin regulation in muscle cells as a component of DGC or as a dystrophin-independent signaling molecule. Our

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results demonstrate that the phosphorylation of  $\alpha$ -syntrophin defines its intracellular localization and the interaction with dystrophin in muscle cells. In addition, we suggest that the phosphorylation of  $\alpha$ -syntrophin by protein kinase C (PKC) regulates the interaction between  $\alpha$ -syntrophin and dystrophin.

## Materials and Methods

### Reagents and antibodies

Staurosporine, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo (2,3-a) pyrrolo (3,4-c)-carbazole (Gö6976), and 12-O-tetradecanoyl-phorbol 13-acetate (TPA) were obtained from Sigma (St. Louis, MO). Anti-dystrophin and anti- $\alpha$ -syntrophin antibodies were provided by Stanley C. Froehner (University of Washington, Seattle, WA). Protein A/G and antibodies of anti- $\text{Na}^+/\text{K}^+$  ATPase, anti-GAPDH, and anti-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). [ $\gamma$ - $^{32}\text{P}$ ]ATP was obtained from DuPont NEN (Boston, MA). All the reagents for cell culture were obtained from Invitrogen (Carlsbad, CA). Bradford assay kit was obtained from Bio-Rad (Hercules, CA).

### Cell culture

C2 or Sol8 myoblasts were cultured as previously described<sup>19</sup>. Cells seeded at a concentration of  $1.0 \times 10^4$  cells/mL were grown in 10% fetal bovine serum-containing Dulbecco's modified Eagle's medium (DMEM) for 3 days (myoblasts, MB). For the induction of differentiation, cells were transferred into 5% horse serum-containing DMEM and incubated for 4 days (myotubes, MT).

### Separation of nuclear and cytoplasmic fractions

The cultured cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested with a scraper. The cells were resuspended in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM  $\text{MgCl}_2$ , 5% glycerol, 1 mM PMSF, 1 mM  $\text{Na}_3\text{VO}_4$ , 10 mM NaF, and 0.1% protease inhibitor cocktail and disrupted by ultrasonication. Protein concentration was determined by the Bradford assay with bovine serum albumin (BSA) as a standard. For the fractionation, equal amounts of total cell lysates (T) were centrifuged at  $25000 \times g$  for 90 min at  $4^\circ\text{C}$ . After centrifugation, the supernatant containing the soluble protein fraction (S) was collected. The pellet was rinsed twice with the lysis buffer and the precipitant proteins were resuspended with 1% Triton X-100 containing buffer. After centrifugation at

$25000 \times g$  for 90 min at  $4^\circ\text{C}$ , the supernatant containing the membrane proteins was the insoluble fraction (I).

### Western blot analysis

Proteins were resolved by 10% or 4-13% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins on the gel were transferred onto polyvinylidene difluoride (PVDF) membranes and the membranes were incubated with the indicated antibodies. After incubation with anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase, the immune reactive protein bands were visualized by enhanced chemiluminescence detection.

### Immunoprecipitation analysis

The cultured MB and MT were washed twice with ice-cold PBS, harvested, and incubated in RIPA buffer (pH 7.4) containing 9.1 mM  $\text{Na}_2\text{HPO}_4$ , 1.7 mM  $\text{NaH}_2\text{PO}_4$ , 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, and 0.1% protease inhibitor cocktail. Cells were then disrupted by ultrasonication. For pre-clearance, 20  $\mu\text{L}$  of protein A/G-agarose beads were incubated with cell suspension (500  $\mu\text{g}/500 \mu\text{L}$ ) for 30 min on ice. The primary antibody (10  $\mu\text{g}$ ) was added to the pre-cleared cell lysates and incubated overnight at  $4^\circ\text{C}$ . Then protein A/G beads (20  $\mu\text{L}$ ) were added and incubated for 2 h at  $4^\circ\text{C}$  with gentle rocking. The immune-complexes were collected by centrifugation and washed 3 times with PBS. The final wash was done with RIPA buffer. The immunoprecipitants were centrifuged at  $10000 \times g$  for 3 min and resuspended in 50  $\mu\text{L}$  of the Laemmli sample buffer. The immune-complexes were separated by SDS-PAGE.

### In vitro phosphorylation assay

Cells treated with or without the indicated reagents were harvested and disrupted in the lysis buffer by ultrasonication. The same amount of proteins (200  $\mu\text{g}/200 \mu\text{L}$ ) was phosphorylated with 10 mM ATP containing 30  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ] ATP at  $30^\circ\text{C}$  for 30 min. Then the lysates were separated in nuclear and cytoplasmic fractions as described above. Each fraction was incubated with dystrophin or  $\alpha$ -syntrophin antibodies overnight at  $4^\circ\text{C}$ . The immune-complexes were incubated with protein A/G solution (20  $\mu\text{L}$ ) for 3 h and precipitated by centrifugation at  $10000 \times g$  for 1 min, followed by washing twice with PBS. The pellet proteins were solubilized with the Laemmli

sampling buffer and separated by 4-13% gradient SDS-PAGE. The gel was then dried and exposed to an X-ray film at  $-70^{\circ}\text{C}$  for 24~72 h. The band intensity on the Coomassie blue stained gel and X-ray film were determined using the Scion image software (Fredrick, MD).

## Results

### The phosphorylated $\alpha$ -syntrophin is localized to the plasma membrane regardless of dystrophin expression

For this study, the C2 cell line isolated from adult mouse skeletal muscle and the Sol8 cell line from mouse oxidative (soleus) muscle were used. The main difference between these cell lines is that Sol8 cells do not express dystrophin during the course of differentiation<sup>20,22</sup>. Both cell lines were cultured as described in the "Materials and Methods". Syntrophins are known as a binding protein of dystrophin and fail to localize at the sarcolemma in the absence of dystrophin<sup>5</sup>. Therefore, we first examined whether the expression and sub-localization of  $\alpha$ -syntrophin are dependent on the expression of dystrophin. The myoblasts and myotubes of C2 and Sol8 cells were fractionated into the soluble fraction containing cytoplasm (S) and the insoluble fraction containing plasma membrane (I). As expected, dystrophin is expressed and located only in the insoluble fraction (I) of the C2 myotubes but not in myoblasts (Fig. 1, left panel). In Sol8 cells, dystrophin was detected neither

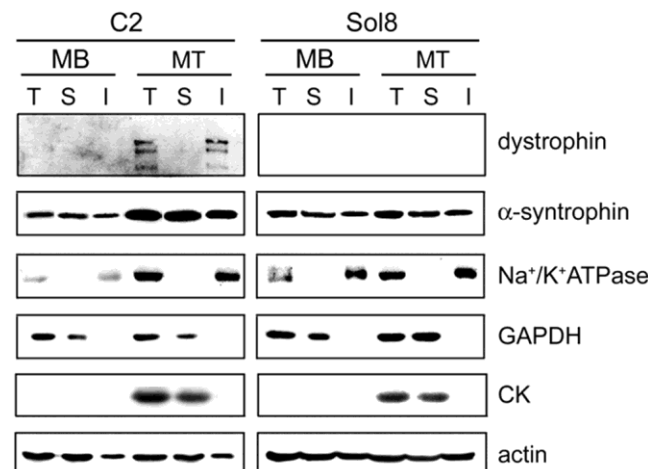


Fig. 1 — Intracellular localization of  $\alpha$ -syntrophin is irrelevant to the expression of dystrophin. MB and MT were disrupted by ultrasonication (total cell lysates, T) and then fractionated into the soluble fraction containing cytoplasm (S) and insoluble fraction containing the plasma membrane (I). The expression of the indicated proteins was detected by western blot.  $\text{Na}^+/\text{K}^+$  ATPase and GAPDH were used as membrane and cytoplasmic marker proteins, respectively

in myoblasts nor myotubes, as is already known<sup>22</sup> (Fig. 1, right panel). In C2 cells, the total expression level of  $\alpha$ -syntrophin in myotubes was higher than that in myoblasts, as we previously reported<sup>20</sup>. The  $\alpha$ -syntrophin was found in both cytoplasm (S) and membrane (I) fractions in myoblasts as well as in myotubes. In contrast, the expression of  $\alpha$ -syntrophin in Sol8 cells was relatively constant over the course of differentiation; there was little increase of expression in myotubes compared to myoblasts. Creatine kinase (CK) was used as a marker protein of myoblast differentiation. Actin was used as a loading control.

Phosphorylation is an important post-translational modification that regulates the localization of proteins<sup>12</sup>. To investigate the relevance between the phosphorylation status of  $\alpha$ -syntrophin and its intracellular localization, an *in vitro* phosphorylation assay was performed (Fig. 2). In C2 myoblasts, the phosphorylated  $\alpha$ -syntrophin was detected only in the insoluble fraction (I) containing plasma membranes but not in the soluble fraction (S) (Fig. 2, left panel). In myotubes, however, phosphorylated  $\alpha$ -syntrophin was not detected in the insoluble fraction (I) despite the protein band on the Coomassie stained gel (arrowhead). The lower band indicated by arrowhead might be IgG heavy chain, phosphorylated by p60src or cytoskeleton D<sup>23</sup>. On the other hand, in Sol8 cells that do not express dystrophin, phosphorylated  $\alpha$ -syntrophin was detected in the insoluble fraction (I), both in myoblasts and myotubes (Fig. 2, right panel). These results show that the phosphorylated  $\alpha$ -syntrophin is accumulated only in the membrane fraction of the muscle cells regardless of the expression of dystrophin.

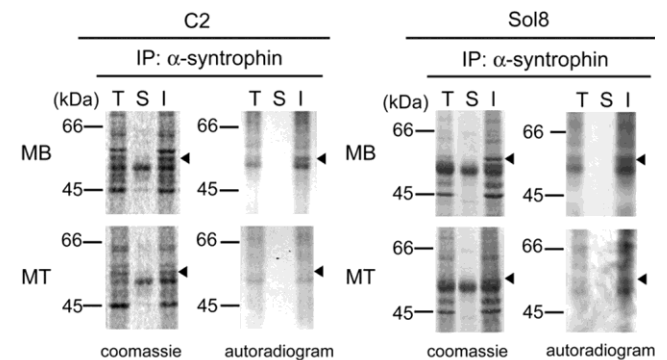


Fig. 2 — Phosphorylated  $\alpha$ -syntrophin is localized to the plasma membrane. The proteins were fractionated into the soluble (S) and insoluble (I) fraction. Each fraction was immunoprecipitated using the anti- $\alpha$ -syntrophin antibody. The immunoprecipitated proteins were separated with SDS-PAGE. The gels were stained with Coomassie brilliant blue R-250 and exposed to X-ray film.  $\alpha$ -Syntrophin is indicated by the arrowhead

### Dystrophin-associated $\alpha$ -syntrophin is not phosphorylated

We then investigated the relevance between the phosphorylation status of  $\alpha$ -syntrophin and interaction with dystrophin. At first, the interaction of dystrophin and  $\alpha$ -syntrophin was confirmed in C2 myoblasts and myotubes by co-immunoprecipitation experiments. As shown in (Fig. 3A), the dystrophin-associated

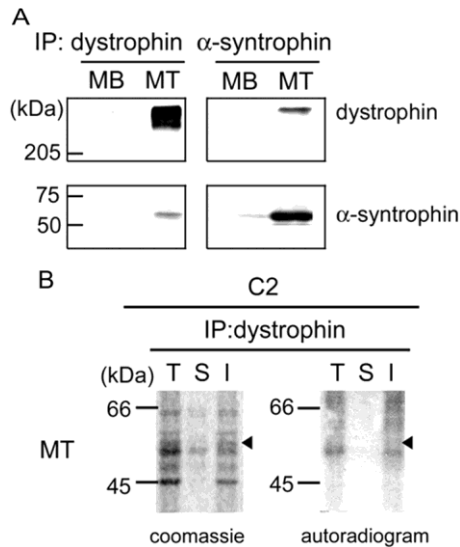


Fig. 3 — Dystrophin-associated  $\alpha$ -syntrophin is not phosphorylated (A) C2 cells were disrupted and immunoprecipitated with anti-dystrophin or anti- $\alpha$ -syntrophin antibodies. The immune complexes were separated by SDS-PAGE. The interaction between  $\alpha$ -syntrophin and dystrophin was detected by western blot with the indicated antibodies; and (B) *In vitro* phosphorylation of the total cell lysate (T) was performed and then fractionated into the soluble (S) and insoluble (I) fractions. Each fraction was immunoprecipitated using the anti-dystrophin antibody.  $\alpha$ -Syntrophin is indicated by the arrowhead

$\alpha$ -syntrophin was detected only in the myotubes. Since the membrane-localized  $\alpha$ -syntrophin was phosphorylated (Fig. 2), we investigated whether the dystrophin-associated  $\alpha$ -syntrophin was also phosphorylated in C2 myotubes. All the proteins immunoprecipitated by anti-dystrophin antibody were visualized by Coomassie staining, and phosphorylation was detected by autoradiography. On the contrary to our expectation, the isotopic bands corresponding to  $\alpha$ -syntrophin (arrowhead in Fig. 3B) were not detected in the insoluble fraction (I), which shows that the  $\alpha$ -syntrophin interacted with dystrophin is not phosphorylated.

### PKC activity is involved in the interaction between dystrophin and $\alpha$ -syntrophin

Based on the finding that intracellular localization of  $\alpha$ -syntrophin and its interaction with dystrophin depends on phosphorylation, we next examined the responsible kinase(s) for the phosphorylation. The possible target sequence(s) for phosphorylation in  $\alpha$ -syntrophin was analyzed by using the NetPhos 2.0<sup>24</sup> server (Fig. 4). NetPhosK 1.0<sup>25</sup> predicted the putative kinases responsible to the phosphorylation for each residue (Table 1). From these results was evident that PKC was the most possible candidate related to the phosphorylation of  $\alpha$ -syntrophin. Therefore, we investigated whether PKC activity was involved in the phosphorylation of  $\alpha$ -syntrophin. C2 myoblasts were treated with or without TPA, a PKC activator. After harvesting, the cell lysates were incubated with staurosporine, a general PKC inhibitor, or Gö6976, a specific inhibitor of  $\text{Ca}^{2+}$ -dependent classical PKC. As

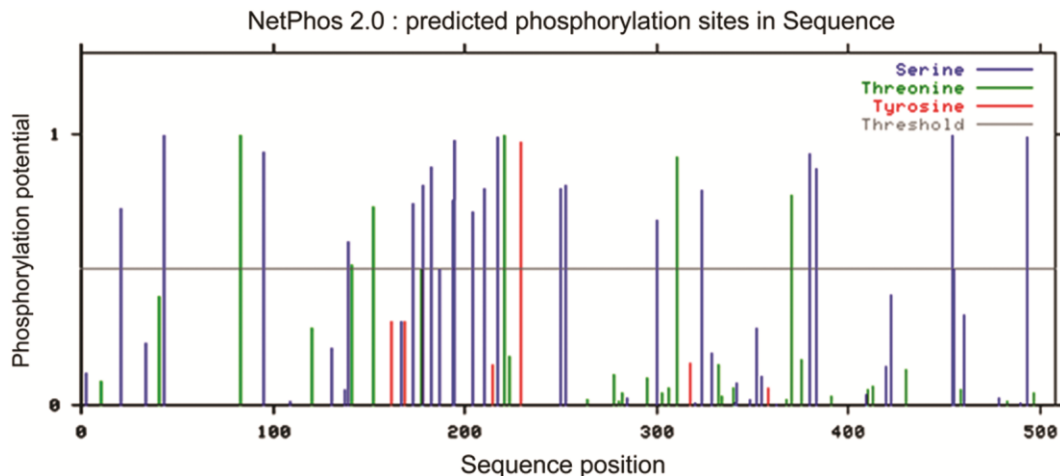


Fig. 4 — Prediction of the phosphorylated residues of  $\alpha$ -syntrophin. Phosphorylation sites in  $\alpha$ -syntrophin were predicted by NetPhos 2.0 Server. X-axis represents the amino acid sequence of  $\alpha$ -syntrophin from N- to C-terminus. Y-axis represents the potentiality of phosphorylation computed by the software. The values above threshold indicate the probability of the corresponding residues to phosphorylation

Table 1 — Putative phosphorylation sites of  $\alpha$ -syntrophin and responsible kinases. Prediction of the putative phosphorylated sites in  $\alpha$ -syntrophin was performed using NetPhosK 1.0 Server. Kinases with the highest score are estimated to phosphorylate the corresponding residues. PKC; Protein kinase A (PKA); Casein kinase II (CK II); Protein kinase G (PKG); Cyclin-dependent kinase 5 (Cdk5); DNA-dependent protein kinase (DNAPK)

Phosphorylation Site	Predicted kinase	Score
Ser 3	PKC	0.86
Ser 34	PKA	0.65
Thr 83	PKC	0.89
Thr 83	PKA	0.63
Ser 109	PKC	0.87
Thr 141	CKII	0.61
Thr 152	PKG	0.64
Ser 187	Cdk5	0.67
Ser 217	PKC	0.83
Thr 221	Cdk5	0.69
Ser 285	DNAPK	0.64
Ser 349	PKC	0.64
Ser 352	PKC	0.75
Thr 368	PKC	0.80
Thr 413	PKC	0.70
Ser 420	PKA	0.65
Ser 422	PKC	0.64
Ser 490	PKC	0.66
Ser 493	PKC	0.79

shown in (Fig. 5), C2 myoblasts incubated with or without 100 nM TPA for 30 min were harvested and disrupted by ultrasonication. Then the cell lysates were incubated with 4 ng/mL of staurosporine or 5 nM of Gö6976 for 30 min at 30°C. Subsequently, the phosphorylated level of  $\alpha$ -syntrophin was significantly decreased by both staurosporine and Gö6976 (39% and 68%, respectively) whereas, it was increased (57%) by TPA. However, the intensity of the protein bands on the Coomassie-stained gel was not significantly changed by those reagents. PKC activator also increased the phosphorylation of  $\alpha$ -syntrophin despite co-treatment with PKC inhibitors. Next, we examined whether PKC activity would affect the expression of  $\alpha$ -syntrophin or its interaction with dystrophin in C2 myotubes. Protein expression of  $\alpha$ -syntrophin was affected neither by staurosporine nor Gö6976 (Fig. 6A). The amount of dystrophin-associated  $\alpha$ -syntrophin, however, was significantly increased by PKC inhibition (Fig. 6B). These results demonstrate that PKC activity affects the interaction between  $\alpha$ -syntrophin and dystrophin without any alteration of the  $\alpha$ -syntrophin expression.

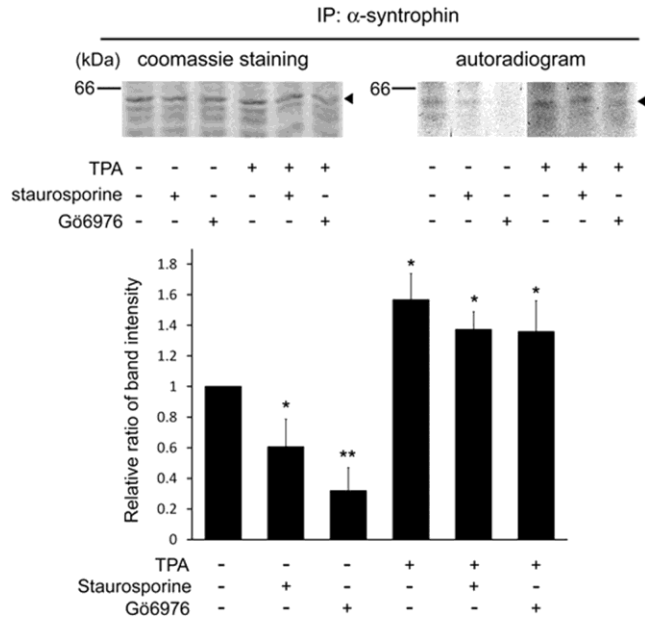


Fig. 5 — PKC is involved in the phosphorylation of  $\alpha$ -syntrophin. The cell lysates were phosphorylated with [ $\gamma$ - $^{32}$ P] ATP and immunoprecipitated with the anti- $\alpha$ -syntrophin antibody. The  $\alpha$ -syntrophin is indicated by the arrowhead. Band intensities of Coomassie staining and autoradiogram were measured using the Scion image and then expressed as a relative ratio of autoradiogram to Coomassie staining. Asterisks indicate a statistical significance compared to the value of the none-treated cells (\* $P$  < 0.05 and \*\* $P$  < 0.01, respectively)

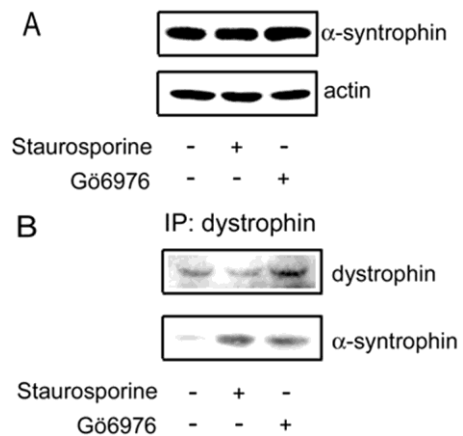


Fig. 6 — The Inhibition of PKC increases the interaction of  $\alpha$ -syntrophin with dystrophin. (A) C2 myotubes were disrupted and incubated with 4 ng/mL of staurosporine or 5 nM of Gö6976 for 30 min at 30°C. The expression level of  $\alpha$ -syntrophin was determined by western blot. Actin was used as a loading control; and (B) The same cell lysates were immunoprecipitated with the anti-dystrophin antibody and the immune complex was separated by SDS-PAGE. The interaction of dystrophin and  $\alpha$ -syntrophin was detected by western blot with the indicated antibodies

## Discussion

Despite the early expression during the course of myoblast differentiation and its key role as a signaling molecule in muscle cells, most studies on  $\alpha$ -syntrophin have focused on its function as a dystrophin-associated protein. Unlike dystrophin, which appears in the relatively later stages of muscle differentiation, a large amount of syntrophin exists in undifferentiated myoblasts<sup>20</sup>. We have previously reported that  $\alpha$ -syntrophin is involved in the myogenin expression during the early stages of differentiation and muscle regeneration and is also required for the hepatocyte growth factor-induced cell migration of the cultured myoblasts<sup>20,21</sup>. In addition, we have shown that  $\alpha$ -syntrophin modulates the concentration of intracellular  $\text{Ca}^{2+}$  and PI3-kinase/Akt signaling pathway under menadione-induced oxidative stress<sup>26</sup>. Furthermore, it was found in our recent study that  $\alpha$ -syntrophin is related to protein stability of catalase and ubiquitin-proteasome system during myoblast differentiation<sup>27</sup>. These results demonstrate that  $\alpha$ -syntrophin can also play an important role as a dystrophin-independent signaling molecule.

As some functions of the protein can be closely related to its subcellular localization as well as to associated proteins<sup>13</sup>, we focused on the localization of  $\alpha$ -syntrophin over the course of myoblast differentiation. Furthermore, we investigated whether the phosphorylation status can influence the localization of  $\alpha$ -syntrophin and the interaction with dystrophin. It was thought that the membrane localization of  $\alpha$ -syntrophin is dependent on the expression of dystrophin as  $\alpha$ -syntrophin did not appear in the sarcolemma of *mdx* mice in which dystrophin was not expressed<sup>5</sup>. However, in the cultured C2 muscle cells,  $\alpha$ -syntrophin was localized to the plasma membrane regardless of dystrophin expression (Fig. 1). These results imply that dystrophin expression is not sufficient for the membrane localization of  $\alpha$ -syntrophin.

Since  $\alpha$ -syntrophin has various functions not only as a DGC component but also as a DGC-independent signaling molecule, it is important to investigate the underlying mechanism of the regulation of  $\alpha$ -syntrophin localization. Protein phosphorylation is an important post-translational modification closely related to protein-protein interaction and intracellular localization<sup>11,12</sup>. Therefore, we investigated the relationship between the phosphorylation status and intracellular localization of  $\alpha$ -syntrophin using an

*in vitro* phosphorylation assay. Interestingly, the  $\alpha$ -syntrophin accumulated in the membrane fraction of the myoblasts was phosphorylated. However, dystrophin-associated  $\alpha$ -syntrophin was not phosphorylated in the myotubes. Based on the results from the NetPhosK server, we next examined whether PKC is responsible for the phosphorylation of  $\alpha$ -syntrophin and its interaction with dystrophin. It was found for the first time that PKC is involved in the phosphorylation of  $\alpha$ -syntrophin and its interaction with dystrophin.

We focused on the activity of PKC because it is closely related to the status of myoblast differentiation and is decreased during the course of differentiation<sup>28-30</sup>. In undifferentiated myoblasts, when the activity of PKC is high, the phosphorylated  $\alpha$ -syntrophin is localized at the peripheral membrane. However, in myotubes, when the PKC activity is relatively decreased, the phosphorylation of  $\alpha$ -syntrophin is also decreased. Meanwhile, when dystrophin expression increases by the course of differentiation<sup>19,20</sup>, the dephosphorylated  $\alpha$ -syntrophin is likely to interact with it, which brings the formation of DGC. It is difficult to assure that PKC is the only regulator of the phosphorylation of  $\alpha$ -syntrophin. Further studies will be required to investigate other kinase(s) or phosphatase(s) related to  $\alpha$ -syntrophin functions.

## Conclusion

The observation in this study demonstrates that phosphorylation of  $\alpha$ -syntrophin by PKC is involved in its intracellular localization and interaction with dystrophin.

## Acknowledgement

This work has been supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No.NRF-2017R1A2B4008562).

## Conflict of Interest

All authors declare no conflict of interest.

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