Biochemistry and Biophysics Reports 9 (2017) 226–231

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Specific titin and myomesin domains stimulate myoblast proliferation

Irina V. Kravchenko, Vladimir A. Furalyov, Vladimir O. Popov*

Federal Research Centre of Biotechnology, Bach Institute of Biochemistry, Russian Academy of Sciences, Leninskiy prospect 33, 119071 Moscow, Russia

ARTICLE INFO

Keywords:
Myoblast proliferation
Titin
Myomesin
Fn type III domain
Ig-like domain
IGF-1

ABSTRACT

Myofibrillar proteins titin and myomesin stimulated myoblast proliferation as determined by MTT-test and labelled thymidine incorporation in the DNA. Specific Fn type III and Ig-like domains of these proteins were able to exert mitogenic effects as well. Proliferative effect of Fn type III domains was highly sensitive to inhibition of Ca^{2+}/calmodulin dependent protein kinase, whereas the effect of Ig-like domains showed greater sensitivity to the inhibition of adenylyl cyclase – cAMP – PKA pathway. IGF-1 autocrine signalling inhibition partially suppressed mitogenic effects revealed by both domain types.

1. Introduction

Investigation of the mechanisms of skeletal muscle hypertrophy development as well as its regeneration after injury is of great interest both from the point of view of fundamental biochemical science as well as possible medical applications. A number of proteins are known to participate in these processes, whereas some of them act as stimulators while others show inhibitory effects [1].

Different splice forms of insulin-like growth factor 1 (IGF-1) play a crucial role in the processes of activation of skeletal muscle growth and regeneration. Two main forms of this growth factor were shown to be involved in these physiological reactions: the major form encoded by IGF-1Ea mRNA [2,3], and a special form encoded by IGF-1Ec mRNA [4,5] often designated as mechano-growth factor (MGF). Expression of both these splice forms increased in response to skeletal muscle injury [6,7] and intensive exercise [8,9] implicating them in the mechanism of the development of skeletal muscle hypertrophy and recovery [10,11].

Earlier we showed that myofibrillar proteins titin and myomesin when released from damaged muscle stimulate the expression of both IGF-1 splice forms [12]. Specific Fn type III and Ig-like domains comprising the structure of titin and myomesin were found to be responsible for this effect [13]. Domains of each type were shown to bind to their own specific receptors and to have different mechanism of action. The effect of Fn type III domains was more sensitive to inhibition of Ca^{2+}/calmodulin dependent protein kinase activity, whereas the effect of Ig-like domains showed greater sensitivity to the inhibition of adenylyl cyclase – cAMP – PKA pathway.

However, a number of questions still remain unanswered. It still remains unknown whether myofibrillar proteins titin and myomesin per se can stimulate myoblast proliferation. If such stimulation indeed takes place, the next question would arise: whether the very same domains that activate IGF-1 splice form expression would stimulate myoblast proliferation. In the case of identity of the domains stimulating myoblast proliferation and activating IGF-1 expression it would be interesting to find out if these domains exert two different physiological effects (cell proliferation and IGF-1 expression) through the same mechanisms of intracellular signalling or through different ones. And at last, the question would arise whether the activation of IGF-1 splice form expression by titin and myomesin domains described earlier plays any role in the stimulation of proliferation by these domains.

Thus the aim of this work was to study the ability of titin and myomesin as well as of their specific domains to stimulate myoblast proliferation and to investigate the signalling pathways responsible for these effects. Here, we provide experimental evidence that titin and myomesin as well as their domains are able to stimulate myoblast proliferation. We have also shown that Fn type III and Ig-like domains render the mitogenic action through different regulatory pathways and that IGF-1 signalling contributes to this mitogenic effect only partially.

2. Material and methods

2.1. Proteins

Titin [14], myomesin [15] and actomyosin [16] were isolated from mouse quadriceps muscle as usual. Purified myomesin showed one band on 7.5% PAAG electrophoresis with SDS and purified titin eluted as one peak during Superose 6 size exclusion chromatography. Bovine serum albumin (BSA), mouse transferrin (all from Sigma) and mouse actomyosin were used as controls.

The DNA sequences (Titin_HUMAN, Q8WZ42 and MYOM1_HUMAN, P52179) encoding titin domains (TA166-167 with

* Corresponding author.
E-mail address: vpopov@inbi.ras.ru (V.O. Popov).

http://dx.doi.org/10.1016/j.bbrep.2016.12.007
Received 5 November 2016; Received in revised form 16 December 2016; Accepted 28 December 2016
Available online 30 December 2016

a. a. 31648–31848, TA170 with a. a. 32047–32143 and TM4 with amino acid residues 33294–33395) and myomesin domains (My2 with a. a. 273–374, My3 with a. a. 409–502, My4 with a. a. 503–605, My5 with a. a. 635–731, My7-My9 with a. a. 935–1227, My11-My13 with a. a. 1352–1666) respectively were amplified by PCR from cDNA libraries. The PCR products were cloned into the pETM11 vector (EMBL), a vector carrying a N-terminal hexa-histidine tag and a tobacco etch virus cleavage site N-terminal to the titin encoding sequence. The constructs were expressed in *Escherichia coli* strain BL21 (DE3) CodonPlus-RIL. The purification protocol included the Ni-NTA affinity chromatography step, removal of the hexa-histidine tag by 6–8 h of incubation with tobacco etch virus protease and a final purification step of size exclusion chromatography (GE, Superdex 75 16/60). All studied protein samples were electrophoretically homogenous.

Protein concentration was determined using the Lowry method with the Bio-Rad DC Protein Assay kit.

2.2. Cell cultures

Primary human and murine myoblast cultures were obtained according to a standard protocol [17,18]. Cells were cultivated in growth medium consisting of DMEM and 10% fetal calf serum (FCS) at 37 °C in a humid atmosphere containing 6% CO_2.

Purity of the myoblast cultures (> 98%) was evaluated by anti-desmin immunostainings.

2.3. MTT assay

Cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)−2−5-diphenyltetrazolium-bromide (MTT) assay and [^{3}H]-thymidine incorporation. MTT assay was routinely employed [19]. Briefly, 2×10^3 murine myoblasts per well or 5×10^3 human ones were plated on a 96-well culturing plate (Corning Costar) for 24 h in DMEM with 10% FCS. To examine the effects of proteins with or without inhibitors on cell proliferation, cells were serum-starved in DMEM supplemented with 0.5% FCS for 10 h. Then investigated proteins with or without inhibitors were added and incubated for 48 h in the same medium. Next the culture medium in wells was replaced for DMEM with 0.5% FCS with 0.5 mg/ml MTT, and cells were incubated for 4 h. The medium from wells was replaced with 150 µl of dimethyl sulfoxide (Sigma-Aldrich), and absorbance at 595 nm was measured using a plate reader (ThermoLabsystems). Each presented point is an averaged data of 8 experiments.

Following inhibitors were used: adenylyl cyclase inhibitor dideoxyadenosine (DDA) at 10 µM concentration; protein kinase A inhibitor Rp-cAMPS at 100 µM; Ca^{2+}/calmodulin dependent protein kinase inhibitor KN93 at 10 µM; IGF-1 receptor inhibitor PQ401 at 2 µM (all from Sigma-Aldrich); antibodies to IGF-1 at 10 µg/ml (R & D).

2.4. [^{3}H]-thymidine incorporation

For determination of [^{3}H]-thymidine incorporation in DNA, 2×10^4 murine myoblasts per well or 4×10^4 human ones were cultured in a 24-well culturing plate (Corning Costar) for 24 h in DMEM with 10% FCS. To examine the effects of proteins with or without inhibitors on cell proliferation, cells were serum-starved in DMEM supplemented with 0.5% FCS for 18 h [20]. Then investigated proteins with or without inhibitors were added and incubated for 24 h in the same medium. Next [^{3}H]-thymidine was added at 20 kBq per well for 18 h. After washing of cells 3 times with phosphate buffer pH 7.2 containing 150 mM NaCl, DNA was precipitated with 10% cold trichloroacetic acid (TCA). The pellet in each well was washed 3 times with 10% TCA and dissolved in 0.5 ml of 0.1 M NaOH, and then solution was neutralized and mixed with 10 ml of liquid scintillator Ultima Gold (Perkin Elmer). The counts per minute (cpm) of the radioactive DNA were counted using a Beckman scintillation counter. Each presented point is averaged data of 6 experiments.

2.5. Statistical analysis

Data are presented as mean ± standard error of the mean. Statistical significance of difference between each experimental group and the control one was determined using two-tailed Student's *t*-test. A result with a P-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Stimulation of myoblast proliferation by titin and myomesin

The ability of titin and myomesin isolated from mouse skeletal muscle to stimulate murine myoblast proliferation was investigated. It was found that both myofibrillar proteins activated myoblast proliferation at 5 µg/ml concentration ($p < 0.01$). The cells treated with myomesin and titin showed 89% and 67% gain against control respectively in the MTT proliferation test (Fig. 1A). On the contrary, the treatment of myoblasts with BSA, mouse transferrin and mouse actomyosin had no statistically significant influence on cell proliferation.

A similar picture was observed in the experiments on incorporation of labelled thymidine in the DNA (Fig. 1B). Myomesin treatment increased [^{3}H]-thymidine incorporation 2.6 times, while titin treatment enhanced it 2.1 times. In both cases differences from the control were statistically reliable ($p < 0.01$). Similar to MTT assay experiments, albumin, transferrin and actomyosin did not stimulate myoblast proliferation.

Study of the concentration dependency of the activation effect revealed that statistically reliable ($p < 0.05$) stimulation of MTT reduction takes place at 2.5 µg/ml concentration of both myofibrillar proteins (Fig. 1C) and stimulation of labelled thymidine incorporation in DNA is observed already at 1 µg/ml protein concentration (Fig. 1D). Values of EC50 determined by two methods for both proteins were in the range 2.5–3.5 µg/ml.

3.2. Ability of different domains to stimulate myoblast proliferation

The ability of specific domains or group of domains comprising titin and myomesin to stimulate human myoblast proliferation was also studied. Among the protein samples tested myomesin domains My5 and My11-13 and titin domains TM4 and TA170 were shown to have mitogenic effect on myoblasts. The treatment of the cells with these domains at 5 µg/ml concentration enhanced MTT reduction statistically reliable ($p < 0.01$) by about 1.5-fold relative to control (Fig. 2A). My2, My3, My4, My7-9 and TA166-167 domains demonstrated no ability to stimulate myoblast proliferation.

A similar picture was observed in the experiment on labelled thymidine incorporation in the DNA (Fig. 2B). Domains activating MTT reduction also stimulated [^{3}H]-thymidine incorporation by 2.1 – 2.4 fold. At the same time domains that were inactive in the experiments with MTT also did not show mitogenic activity in experiments with labelled thymidine.

Domains stimulating myoblast proliferation belong to two different structural types: My5 and TA170 have the fold similar to Fn type III domains whereas My11-13 and TM4 – to Ig-like domains. Concentration dependency of the stimulation effect was investigated for all the four domains. It was found that statistically reliable stimulation of MTT reduction takes place at 2.5 µg/ml concentration of domains (Fig. 2C) and stimulation of the labelled thymidine incorporation in DNA is observed at 1 µg/ml protein concentration (Fig. 2D). EC50 for Fn type III domains were approximately 2.5 µg/ml, and EC50 for Ig-like domains were about 3.5 µg/ml.