Specific titin and myomesin domains stimulate myoblast proliferation

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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-1Ec 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 [4,5] 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 Ca2+/calmodulin dependent protein kinase, whereas the effect of Ig-like domains showed greater sensitivity to the inhibition of adenyl cyclase – cAMP – PKA pathway. However, a number of questions still remained 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 MYOM1_HUMAN, P52179) encoding titin and myomesin domains were used as controls.

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2.4. $[^3H]$-thymidine incorporation from Sigma-Aldrich); antibodies to IGF-1 at 10

2.3. MTT assay

5-diphenyltetrazolium-bromide (MTT) assay and $[^3H]$-thymidine incorporation. MTT assay was routinely employed[19]. Briefly, $2 \times 10^4$ murine myoblasts per well or $5 \times 10^3$ 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 18 h[20]. 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 with DMEM for 10% 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 dideoxymycosin (DDA) at 10–20 μM; 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. $[^3H]$-thymidine incorporation

For determination of $[^3H]$-thymidine incorporation in DNA, $2 \times 10^4$ murine myoblasts per well or $4 \times 10^4$ 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 $[^3H]$-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 $[^3H]$-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 $[^3H]$-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.
3.3. Different sensitivity of mitogenic effects of Fn type III domains and Ig-like domains to the inhibitors of intracellular signalling pathways

Next the sensitivity of the mitogenic effect of different domain types towards the inhibitors of major signalling pathways was investigated. All the inhibitors under investigation were not cytotoxic to human myoblasts and did not decrease MTT reduction (Supplement Fig. 1) and [3H]-thymidine incorporation in the DNA (Supplement Fig. 2) in the concentration range studied.

The effects exerted by Fn type III domains and Ig-like domains appeared to differ significantly in their sensitivity towards the action of inhibitors of diverse signalling cascades. Proliferative effect of Fn type III domains My5 and TA170 was strongly inhibited by Ca2+/calmodulin dependent protein kinase inhibitor (p < 0.05). KN93 inhibitor decreased MTT reduction by myoblast cultures to 72–75% of the level observed without the inhibitor (Fig. 3A). In the experiments with [3H]-thymidine incorporation inhibitory effect was pronounced even stronger (p < 0.01): mitogenic effect of Fn type III domains decreased to 59–61% of the level observed without inhibitor (Fig. 3B).

At the same time proliferative effect of Fn type III domains showed little sensitivity to the inhibitors of adenyl cyclase and protein kinase A. DDA and Rp-cAMPS impaired MTT reduction by myoblast cultures treated with these domains as little as to 93–95% of the level observed without inhibitor. These effects were manifested as a trend only (0.1 > p > 0.05). Inhibition of [3H]-thymidine incorporation in the DNA decreased to 75–79% of the level observed without the inhibitor and was also only slightly pronounced, though statistically reliable (p < 0.05).

The character of inhibition patterns for the Ig-like domain mitogenic effects was quite different. Proliferative action of My11-13 and TM4 domains was strongly depressed by inhibitors of adenyl cyclase and protein kinase A. DDA and Rp-cAMPS lowered MTT reduction by myoblast cultures treated with these domains (p < 0.05) to 76–78% of the level observed without the inhibitor. Inhibitory action of these compounds was even more pronounced in the experiments with [3H]-thymidine incorporation (p < 0.01): mitogenic effect of Ig-like domains was decreased to 58–60% of the level observed without the inhibitor. In all cases the inhibition of domain mitogenic effects was statistically reliable.

Meanwhile proliferative effect of Ig-like domains was of little sensitivity to the inhibitor of Ca2+/calmodulin dependent protein kinase. KN93 decreased MTT reduction by myoblast cultures treated with these domains to 94–95% of the level observed without the inhibitor. These effects were manifested as a trend only (0.1 > p > 0.05). Inhibition of [3H]-thymidine incorporation in DNA was also slightly pronounced though statistically reliable (p < 0.05) –76–77% of the level observed without inhibitor.

Inhibition of IGF-1 signalling results in statistically reliable (p < 0.05) decrease of the mitogenic effects exerted by both domain types (Fig. 3A and B). Neutralizing antibodies to this growth factor as well as PQ401 known as the inhibitor of IGF-1 receptor lowered MTT reduction by myoblast cultures treated with domains of both types to 82–87% of the level observed without inhibitor. These inhibitors decrease labelled thymidine incorporation in DNA also: mitogenic effect of domains was decreased to 65–68%.

Fig. 1. Induction of the proliferation of murine myoblasts by titin and myomesin. Murine myoblasts were incubated with tested proteins taken at 5 μg/ml concentration (A, B) or with myomesin and titin taken at concentrations from 0 to 25 μg/ml (C, D). MTT-test (A, C; n=8) and [3H]-thymidine incorporation in DNA measurement (B, D; n=6) were performed after 48 h and 24 h incubation, respectively. Graph represents the mean ± s.e. Significantly different from control values: **p < 0.01.
**incorporation in DNA measurement (B, D; proteins, in particular titin and myomesin, as well as their specific domains, can stimulate expression of IGF-1 splice forms. However, it remained unclear whether these proteins were able to activate myoblast proliferation as well. Here we report the ability of isolated and purified titin and myomesin as well as some of their domains to stimulate MTT reduction and [3H]-thymidine incorporation in the DNA by myoblast cultures. Mitogenic properties were exhibited both by Fn type III and Ig-like domains. It should be emphasized that out of all the studied domains only those that activated IGF-1 splice forms expression were also able to stimulate myoblast proliferation [13]. EC50 values for mitogenic as well as IGF-1 expression stimulation effects were in the range of micrograms per ml.

Similarity of the EC50 values for the proteins significantly differing in molecular weight (Mw of titin as isolated was in the range of 2–3 MDa, myomesin has a molecular weight of 165 kDa, while individual domains have Mw of 10–30 kDa) could be explained by the different content of the active domains comprising individual proteins. EC50 of 2 μg/ml corresponds to 100 nM for a protein of 20 kDa (domain), 10 nM for a protein of 200 kDa (myomesin) and 1 nM for a protein of 2 MDa (titin). However the number of potentially active domains in all these proteins is significantly different and ranges from 1 (individual domain) to several (myomesin) and most probably to several dozen in titin. Thus even a relatively low value of a binding constant for the individual protein fragment/domain, about 100 nM, can result in a substantial stimulating effects originating from myofibrillar proteins comprising large number of such domains.

Earlier we showed that stimulation of MGF and IGF-1 synthesis induced by Fn type III domains was more sensitive to the inhibition of Ca2+/calmodulin dependent protein kinase activity, whereas the effect of Ig-like domains showed greater sensitivity to the inhibition of adenyl cyclase – cAMP – PKA pathway [13]. In the present work we show that stimulation of myoblast proliferation induced by domains of these two types demonstrates the same pattern of sensitivity towards inhibitors of the signalling pathways. Mitogenic effects of Fn type III domains were drastically reduced by KN93 though quite weakly by DDA and Rp-cAMPS, whereas effects exerted by Ig-like domains were considerably lowered by DDA and Rp-cAMPS and to much lesser degree by KN93.

Inhibition analysis reveals that Ig-like domains My11-13 and TM4 activate myoblast proliferation via stimulation of adenyl cyclase and protein kinase A. Mitogenic effect of this signalling pathway activation on muscle cell is well documented. Increase of cAMP level was shown to stimulate the proliferation of myoblasts [21], whereas transcription factor CREB was established to take part in the process of skeletal muscle regeneration [22]. It is more interesting to note that described CREB activation was induced by compounds released from the damaged muscle. These compounds were not identified. However the results of the present work allow to propose that it could be titin and/or myomesin or fragments thereof, e.g. some Ig-like domains comprising their structure.

Fn type III domains My5 and TA170 activate myoblast proliferation via stimulation of Ca2+/calmodulin dependent protein kinase. Mitogenic action of the activation of this enzyme was described also, though not on skeletal muscle cells, but on thyroid cells [23], fibroblasts [24] and smooth muscle cells [25].

It will be of great interest to investigate the downstream targets of protein kinase A and Ca2+/calmodulin dependent protein kinase as well as the possible points of convergence of the two signal cascades. It

**Fig. 2.** Induction of the proliferation of human myoblasts by specific titin and myomesin domains. Human myoblasts were incubated with tested myofibrillar protein domains taken at 5 μg/ml concentration (A, B) or with domains My5, My11-13, TM4 and TA170 taken at concentrations from 0 to 25 μg/ml (C, D). MTT-test (A, C; n=8) and [3H]-thymidine incorporation in DNA measurement (B, D; n=6) were performed after 48 h and 24 h incubation, respectively. Graph represents the mean ± s.e. Significantly different from control values: **p < 0.01.
may be supposed that the most probable PKA target is a transcription factor CREB involved in the stimulation of proliferation of many different cell types [26]. Regarding proteins involved in transducing a mitogenic signal from Ca²⁺/calmodulin dependent protein kinase, it is a challenge to make reasonable assumptions about their nature. Among the targets of this enzyme, there are several proteins that can stimulate cell proliferation including protein kinase Akt [27], histone deacetylases [28] and aforementioned CREB protein [29]. Nevertheless, it might be inferred that the transcription factor CREB is involved in both signalling pathways. In our previous work describing the induction of MGF synthesis by titin and myomesin domains [13], we have shown that the transcription factor CREB is involved in proliferative effects of titin and myomesin domains. In the present work we describe the mitogenic effects of titin and myomesin domains that makes this approach even more viable.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.12.007.

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