as a myostatin-binding protein [8]. FLRG was first identified as a molecule located near the chromosomal breakpoint in human B cell leukaemia [29]. FLRG has a similar domain structure to follistatin, and also has a similar binding specificity [30].

As chemical TGF-β inhibitors, SB431542 and SB505124 were developed [31,32]. SB431542 and SB505124 are selective inhibitors of TGF-β type I receptors, ALK4, ALK5 and ALK7 [31,32]. These compounds are not specific and inhibit TGF-β, activins, myostatin and nodal with similar potency. Various therapeutic strategies for muscular dystrophy by myostatin inhibition are underway.

3.2 Sarcopenia, disuse atrophy and cachexia

In addition to genetic disorders such as muscular dystrophy, muscles are affected by ageing (sarcopenia), denervation, cachexia, immobilisation by disability, and stroke. Atrophying muscles show increased rates of protein degradation over new protein synthesis and regeneration. The precise molecular mechanism of disuse muscle atrophy was not fully understood until recently. Decreased activity of the IGF-1/phosphatidylinositol 3-kinase (PI3K)/Akt kinase pathway leads to muscle atrophy [33]. Recent investigation revealed that FOXO and related forkhead transcription factors act as cofactors of nuclear receptors and play a major role in muscle atrophy by inducing atrophy-related ubiquitin ligase atrogin-1, leading to growth suppression and protein degradation [34,35]. Akt kinase activated by IGF-1 phosphorylates the FOXO family of transcription factors, leading to sequestration of FOXO factors in the cytoplasm from the nucleus of the target cells [34,35]. Muscle atrophy could be prevented by modulating the IGF-1/PI3K/Akt pathway and cellular localisation and phosphorylation of FOXO factors. Interestingly, interaction of forkhead transcription factors such as FAST1 (FOXH1) and the TGF-β downstream molecule Smad has been characterised. Smad proteins activated by TGF-β form a complex with FOXO proteins to activate p21, which is involved in growth suppression. This pathway is negatively controlled by the PI3K pathway, an inhibitor of FOXO nuclear localisation [36]. Thus, it is likely that FOXO transcription factors act at the crossroads of skeletal muscle growth and proliferation, in response to both the IGF pathway and myostatin pathway [36].

Several reports showed that the level of myostatin increased in human ageing associated with muscle wasting, and may be a biomarker of age-associated muscle wasting and cachexia [37-39]. Even with prolonged inhibition of myostatin in senescent mice, skeletal muscle is still capable of regeneration [40]. Thus, myostatin inhibition would be beneficial for sarcopenia and disuse atrophy.

Cachexia occurs in the advanced phase of cancer or severe infection. Myostatin administration *in vivo* induced severe weight loss and decreased muscle mass that is reminiscent of cachexia [41]. Among TGF-β family members, activins are involved in hepatic cachexia with severe weight loss [42]. Thus, either myostatin blockage or activin inhibition would be beneficial for preventing cachexia. In fact, administration of follistatin, a potent antagonist of myostatin and activin, has been shown to be effective for preventing myostatin-induced cachexia [41]. As no clinically effective drugs for cachexia have yet been developed, myostatin and activins would be novel drug targets for cachexia [1,2].

3.3 Neurogenic muscle atrophy

Skeletal muscles receive neuronal inputs and are affected by neuronal disorders. ALS, also known as Lou Gehrig's disease, is an incurable fatal neuromuscular disease characterised by progressive muscle weakness and paralysis. The disease is caused by the selective loss of motor neurons in the brain and spinal cord. Motor neurons, which control the movement of voluntary muscles, deteriorate and eventually die. As muscles no longer receive the signals from neurons, they gradually weaken and become atrophied. Various types of neurodegenerative disease, such as Parkinson's disease, Huntington's chorea and spinocerebellar degeneration, affect skeletal muscle via neurodegenerative pathways.

Peptide growth factors, such as IGF, hepatocyte growth factor and nerve growth factor, are promising interventions for treatment. In the case of IGF-1, retrograde delivery from skeletal muscle to motor neurons by adeno-associated virus prolonged neuron survival and delayed disease onset and progression [43]. Whether myostatin inhibition is effective for neurogenic muscle atrophy is unknown at present.

Activins and BMPs are involved in the development and survival of specific neurons. Activins are expressed in a subset of spinal motor neurons, sensory neurons and central nervous system neurons, and affect neurotransmitter expression [44]. Activin is essential for neuroprotection in the hippocampus [45]. As BMP/activin signals are involved in the development of NMJ structure and specify neuronal cell types and survival in spinal cord [4,12], modulation either of the activin or BMP signal would be favourable for survival of specific neurons.

3.4 Myosarcomas

Although malignancy of muscles is rare, rhabdomyosarcomas are one of the most common solid tumours of childhood. Interestingly, overexpression of myostatin is a common feature of rhabdomyosarcoma. An autocrine myostatin loop contributed to maintain rhabdomyosarcoma cells in an undifferentiated stage, and inhibition of myostatin enhanced differentiation, indicating that new therapeutic approaches could be developed for the treatment of rhabdomyosarcoma by inactivation of myostatin [46]. Unlike in normal myoblasts, signalling of myostatin does not rely on the p21 or Rb pathways, indicating that myostatin signalling is dependent on the differentiation state of cell types [47]. Histone deacetylase inhibitors increased muscle cell size by the induction of follistatin, a potent myostatin and activin antagonist [48]. Thus, chemicals that induce myostatin inhibitors could be used as drugs for rhabdomyosarcoma.

Table 1. Muscular disorders and myostatin.

Disease	Mode of inheritance	Gene locus	Gene defect	Myostatin blockage	Reference
Muscular dystrophies (selected)					
Duchenne	XR	Xp21	Dystrophin	Effective in *mdx* mouse	[19-21]
LGMD1C	AD	3p25	Caveolin-3	ND	
LGMD2A	AR	15q15	Calpain-3	ND	
LGMD2B	AR	2p13	Dysferlin	ND	
LGMD2E	AR	4q12	β-Sarcoglycan	ND	
MDC1A	AR	6q22	Laminin α-2	Not effective in *dy* mouse	[58]
Fukuyama	AR	9q31-33	Fukutin	ND	
Disease		**Overproduction**		**Myostatin blockage**	**Reference**
Myosarcoma					
Rhabdomyosarcoma		myostatin		Effective *in vitro*	[46-48]

AD: Autosomal dominant; AR: Autosomal recessive; LGMD: Limb-girdle muscular dystrophy; MDC: Muscular dystrophy congenital type; ND: Not determined; XR: X-linked recessive.

3.5 Diabetes mellitus/obesity

Metabolic syndrome includes obesity, high blood glucose, hyperlipidaemia, hypercholesterolemia and hypertension, and is a life-threatening disease. Adipocytes and myocytes are derived from common precursor cells, and both cell types secrete hormones that regulate homeostasis of skeletal muscle and adipose tissues. Thus, understanding the regulatory mechanisms that control adipocytes and myoblasts is crucial for understanding the pathophysiology and developing therapies for diabetes/obesity. Interestingly, loss of myostatin not only increases muscle mass, but also prevents an age-related increase in the total mass of adipose tissues. Myostatin-deficient mice showed a reduction in fat accumulation with increasing age [49]. Levels of essential transcription factors such as C/EBPα and PPARγ were reduced in myostatin knockout mice [50]. Conversely, myostatin transgenic mice that overexpressed myostatin in the skeletal muscle had decreased muscle mass and increased fat mass in male mice [51].

Inhibition of myostatin activity in diabetes model mice, such as agouti lethal yellow and $Lep^{ob/ob}$, partially suppressed the obese and diabetic phenotypes. Thus, myostatin could be a novel target for diabetes mellitus [49]. Several *in vitro* studies showed that myostatin inhibited both myogenesis and adipogenesis, whereas one study reported that myostatin inhibited myogenesis and promoted adipogenesis in mesenchymal multipotent cells [24,52]. Thus, the mechanisms by which the number of adipocytes is reduced in myostatin knockout mice are not clear. As adipocytes and myocytes are derived from common mesenchymal progenitors, a massive increase of skeletal muscle *in vivo* by myostatin inhibition may reduce the number of reservoir cells destined to become adipocyte precursor cells in myostatin knockout mice. It is also likely that skeletal muscles with increased mass may secrete factors that inhibit adipocyte growth.

In summary, in addition to adipocytokines, such as leptin and adiponectin, TGF-β family members, including myostatin, are involved in glucose homeostasis and diabetes, and are potential targets for diabetes treatment [53].

4. Biological therapies for muscular disorders

Towards intractable muscular disorders such as muscular dystrophies, no specific therapies have been developed yet. Although many defective genes for various types of muscular dystrophy have been elucidated, therapies for muscular dystrophies have not been realised to date. As described in this review, myostatin blockage is one of the realistic therapies for muscular dystrophy, especially for DMD (**Table 1**). At present, the biosafety and effectiveness of myostatin inhibitors are under investigation in clinical trial studies. A myostatin antibody called MYO-029 is in Phase I/II studies in the US to investigate its effects on > 100 patients suffering from adult-type muscular dystrophies, including BMD, FSHD and LGMD. The results of these clinical trials will be made public in late 2006 (see [101] for details) [54]. In addition to muscular dystrophy, myosarcoma and neurogenic muscle atrophy may be treatable by myostatin blockage.

Two different muscle fibres, type I fibre (red muscle) and type II fibre (white muscle), exist and have different functions. As indicated in section 2, myostatin inhibition in cattle increased the relative percentage of type II fibres [11]. In accordance with this finding, one report indicated that myostatin was a muscle-wasting factor contributing to type 2B and 2A atrophy [55]. By contrast, when FOXO was overexpressed in skeletal muscle, type I fibre in skeletal muscle dramatically decreased [56]. Thus, modulation of the myostatin pathway and FOXO pathway affects both skeletal muscle mass/atrophy and muscle fibre

types. In pathological states, skeletal muscle mass and fibre composition are altered. For example, the number of type I fibres is reduced in obesity [57]. Clinical regulation of muscle mass and even fibre composition may become realistic in the future.

5. Expert opinion and conclusion

Myostatin and BMPs are involved in skeletal myogenesis and homeostasis. Myostatin inhibition is a promising and realistic therapy for muscular disorders. We should keep in mind that although myostatin inhibition favours an increase in muscle mass, it may decrease the reservoir of skeletal muscle stem cells. Experimentally, skeletal muscles regenerate robustly after chronic and acute injury in mice with prolonged absence of myostatin, suggesting that myostatin inhibition favours skeletal regeneration for a prolonged time [40]. However, as regeneration of skeletal muscle in mice is intrinsically more active compared with humans, it remains to be determined whether skeletal muscles are able to regenerate to a favourable degree after myostatin inhibition in humans.

We should be careful about choosing proper types of muscular dystrophies for myostatin inhibition (**Table 1**). In *mdx* mouse, which is the DMD model mouse, myostatin inhibition therapy was reported to be effective [19-21]. By contrast, elimination of myostatin inhibition in *dy* mice, which is the laminin-α-deficient mouse model of muscular dystrophy, did not combat muscular dystrophy but increased postnatal lethality [58] (**Table 1**). *dy* Mice showed severe muscle atrophy compared with *mdx* mice. As the number of myoblasts and skeletal muscle stem cells that responded to myostatin was severely reduced in *dy* mice, myostatin inhibition may not be a proper way to treat *dy* mice. Thus, it is important to determine which types of muscular dystrophies and disease states are suitable for myostatin inhibition therapy. We should also take into consideration that feedback regulation and bioavailability of myostatin are under tight regulation by multiple mechanisms such as protease sensitivity [2,59]. One of the key issues of myostatin inhibition therapy for muscular dystrophy is that defective genes are not recovered by myostatin inhibition. Thus, other approaches such as gene therapy to restore normal genes and/or cell transplantation therapy should be combined with myostatin inhibition therapy.

In summary, myostatin inhibition could be effective not only for muscular dystrophy, but also for other muscular disorders, such as disuse atrophy and even neurogenic muscle atrophy. Thus, under careful consideration to avoid undesirable side effects, myostatin inhibition therapy could be applicable to multiple human muscular disorders.

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Characterization of isoforms of activin receptor-interacting protein 2 that augment activin signaling

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Abstract

Activin type II receptors (ActRIIs) including ActRIIA and ActRIIB are serine/threonine kinase receptors that form complexes with type I receptors to transmit intracellular signaling of activins, nodal, myostatin and a subset of bone morphogenetic proteins. ActRIIs are unique among serine/threonine kinase receptors in that they associate with proteins having PSD-95, Discs large and ZO-1 (PDZ) domains. In our previous studies, we reported specific interactions of ActRIIs with two independent PDZ proteins named activin receptor-interacting proteins 1 and 2 (ARIP1 and ARIP2). Overexpression of both ARIP1 and ARIP2 reduce activin-induced transcription. Here, we report the isolation of two isoforms of ARIP2 named ARIP2b and 2c. ARIP2, ARIP2b and ARIP2c recognize COOH-terminal residues of ActRIIA that match a PDZ-binding consensus motif. ARIP2 and its isoforms have one PDZ domain in the NH_2-terminal region, and interact with ActRIIA. Although PDZ domains containing GLGF motifs of ARIP2b and 2c are identical to that of ARIP2, their COOH-terminal sequences differ from that of ARIP2. Interestingly, unlike ARIP2, overexpression of ARIP2b or 2c did not affect ActRIIA internalization. ARIP2b/2c inhibit inhibitory actions of ARIP2 on activin signaling. ARIP2 is widely distributed in mouse tissues. ARIP2b/2c is expressed in more restricted tissues such as heart, brain, kidneys and liver. Our results indicate that although both ARIP2 and ARIP2b/2c interact with activin receptors, they regulate ActRIIA function in a different manner.

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Introduction

Activins belong to the transforming growth factor-β (TGF-β) superfamily, and signaling occurs via two types of membrane-bound receptor complexes (Attisano *et al.* 1996, Tsuchida *et al.* 2001). The two types of activin receptors, i.e. type I and type II, are serine/threonine kinase receptors. The two subtypes of the activin type II receptor, ActRIIA and IIB, are encoded by separate genes (Sugino & Tsuchida 2000). In addition, two spliced variants of ActRIIA including ActRIIA-N (Mathews & Vale 1991, Shoji *et al.* 1998), and five spliced variants of ActRIIB have been detected (Attisano *et al.* 1992, Ethier *et al.* 1997). Ligands that bind to ActRIIs include activins, myostatin, nodal and bone morphogenetic protein 7 (BMP-7). Direct binding of these ligands to ActRIIs promotes the recruitment of type I receptors to the complex, which in turn results in phosphorylation of type I receptors, activation of type I receptors, and downstream propagation of the signal (Mathews 1994, Sugino & Tsuchida 2000, Tsuchida 2004).

The significance of the different ActRIIs is yet to be determined. Signal transduction via transmembrane receptors is regulated by their interaction with PDZ domain-containing molecules. PDZ domain-containing proteins play an important role in assenibly of receptors and signaling molecules near submembranous regions (Fanning & Anderson 1999). To determine the specific functions of ActRIIs in regulating activin signal transduction, we searched for cytoplasnic proteins that interacted with the receptors. We identified two cytoplasmic proteins named activin receptor-interacting proteins 1 and 2 (ARIP1 and ARIP2). ARIP1 showed multiple protein–protein interacting domains including two WW domains which interacted with Smad3, and five to six PDZ domains which interacted with ActRIIs, phosphatase and

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tensin homologue deleted on chromosome 10 (PTEN) and β-catenin (Hirao *et al.* 1998, Shoji *et al.* 2000). ARIP2 possesses one PDZ domain which interacts with ActRIIA. ARIP2 also associates with Ral binding protein 1 (RalBP1), and assembles into a ternary complex that includes ARIP2, ActRII and RalBP1 (Matsuzaki *et al.* 2002). Overexpression of ARIP1 and ARIP2 inhibits activin-induced transcriptional activity in a dose-dependent manner (Shoji *et al.* 2000, Matsuzaki *et al.* 2002). In the present study, we report the identification of two isoforms of ARIP2, named ARIP2b and 2c. Similar to ARIP2, ARIP2b and 2c have only one PDZ domain, which specifically interacts with ActRIIA. However, even though ARIP2b and 2c were structural variants of ARIP2, they showed different functions. Overexpression of ARIP2b and 2c resulted in increased activin-induced signaling. Furthermore, we found that ARIP2b and 2c increased ActRIIA expression at the cell surface without affecting internalization of ActRIIA, whereas ARIP2 was involved in endocytosis of ActRIIA (Matsuzaki *et al.* 2002). Thus, multiple ARIP2 proteins can interact with ActRII to regulate its signaling and trafficking in a different manner.

Materials and Methods

cDNA cloning

Yeast two-hybrid screening was performed using a commercially available system (Matchmaker Two-Hybrid System 2; Clontech) in accordance with the manufacturer's protocol. Approximately 4×10^6 clones of a mouse brain cDNA library were screened using the bait construct pAS-ActRIIA, which contains nucleotide sequences for the entire cytoplasmic region of mouse ActRIIA (554–1995) fused to a GAL4 DNA binding domain (Shoji *et al.* 2000, Tsuchida *et al.* 2001). One clone obtained by the yeast two-hybrid screening was named #YA-1. A mouse brain cDNA library in the lambda ZAPII vector (Stratagene, La Jolla, CA, USA) was screened using the #YA-1 cDNA as probe. ARIP2 cDNA was identified by screening as described previously (Matsuzaki *et al.* 2002). Additional clones encoding full-length ARIP2b and 2c sequences were identified by screening the brain library using full-length ARIP2 cDNA as hybridization probe.

DNA constructs

DNA constructs for the yeast two-hybrid screening were made using either the plasmid pAS2–1 to express the fusion protein with the GAL4 DNA-binding domain, or the plasmid pACT2 to express the fusion protein with the GAL4 activation domain. pAS-ActRIIA was made by introducing nucleotides 554–1995 of mouse ActRIIA (Mathews & Vale 1991) into pAS2–1. For the mammalian two-hybrid assay, DNA was constructed using the plasmid pBIND to express the fusion protein with the GAL4 DNA-binding domain, and the plasmid pACT to express the fusion protein with the VP16 activation domain. To make pBIND-ActRIIA, cDNA fragments encoding the entire cytoplasmic regions of ActRIIA were subcloned into pBIND. Using PCR-based mutagenesis, mutations in the COOH terminus of ActRIIA were generated by replacing appropriate nucleotides with mutated oligonucleotides (Shoji *et al.* 2000). Other receptor constructs have been described previously (Shoji *et al.* 2000). To make pACT-ARIP2b and 2c, cDNA fragments covering coding regions (nucleotides 453–758 of ARIP2b, and nucleotides 1–357 of ARIP2c) were prepared and subcloned into pACT. To make pACT-ARIP2cΔC and pACT-ARIP2cΔPDZ, cDNA fragments composed of nucleotides 1–297 and 298–357 of ARIP2c respectively, were prepared by PCR and ligated into pACT. Expression constructs of ARIP2b and 2c were made by subcloning nucleotides 453–758 of ARIP2b and nucleotides 1–357 of ARIP2c into pCIneo and pcDNA3 respectively. FLAG-tagged ARIP2b and 2c were subcloned into pcDNA3. Full length ActRIIA cDNA was subcloned into pcDLSRα. Myc-tagged ActRIIA cDNA in pcDNA3 was made by incorporating 7 myc epitopes after the arginine residue (amino acid number 24) of ActRIIA cDNA in pcDNA3.

Yeast and mammalian two-hybrid analysis

Yeast two-hybrid assays were performed using a commercially available kit (Matchmaker Two-Hybrid System 2; Clontech) in accordance with the manufacturer's protocol. Mammalian two-hybrid assays were performed using the CheckMate Mammalian Two-Hybrid System (Promega). In brief, Chinese hamster ovary K1 (CHO-K1) cells were co-transfected with appropriate plasmids, a cytomegalovirus promoter-driven β-galactosidase (CMV-β-gal), and the reporter plasmid pG5 luc, which drives the luciferase gene under the control of the GAL4-responsive promoter. Luciferase activity was measured and normalized against the level of β-gal activity as described previously (Tsuchida *et al.* 1995).

Immunoprecipitation and Western blotting

Interactions of full-length ARIP2b or 2c with ActRIIA were studied. Using the calcium phosphate precipitation method, COS-7 cells or HEK 293 cells were transfected either with pcDNA3-FLAG-ARIP2b or 2c alone or co-transfected with pcDNA3-FLAG-ARIP2b (or 2c) and pcDLSRα-ActRIIA. Two days after transfection, cells were harvested using lysis buffer (1% Nonidet P-40, 50 mM Tris–HCl (pH 7·5), 150 mM NaCl, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 μg/ml leupeptin, and 1 μg/ml aprotinin). The lysate was cleared

by centrifugation, and proteins were immunoprecipitated by adding a rabbit anti-ActRIIA polyclonal antibody (R&D Systems, Minneapolis, MN, USA), or anti-FLAG M2 monoclonal antibody (Sigma). Samples were then incubated with protein G-sepharose (Amersham Pharmacia Biotech) at 4 °C for 2 h. Precipitated proteins were fractionated by SDS-PAGE, and transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were initially incubated with an anti-FLAG antibody (1:1000 dilution), and then with a horseradish peroxidase-conjugated secondary antibody. Labeled proteins were detected by chemiluminescence (ECL-Plus; Amersham Pharmacia Biotech) (Bao *et al.* 2005). To study interactions of ARIP2 or ARIP2c with RalBP1, FLAG-ARIP2 (or 2c) and myc-tagged RalBP1 were co-transfected into HEK 293 cells and the lysate was immunoprecipitated with an anti-FLAG antibody and immunodetected using myc antibody (1:1000 dilution).

RNA extraction and RT-PCR

Total RNA from mouse tissues was extracted using the TRIzol reagent according to the manufacturer's protocol (Invitrogen). Polyadenylated RNA was further purified using oligo (dT) latex beads (Takara, Tokyo, Japan). RNA samples were then reverse transcribed with oligo dT primer using SUPERSCRIPT II reverse transcriptase (Gibco-BRL). The ARIP2 primer set included the sense primer 5′-GGA GAG CAG TCA GAT ATG AAC G-3′, and the antisense primer 5′-CAC GAA GAC CAA AAG AAC CTC CAA C-3′, and the ARIP2c primer set included the sense primer 5′-GGA GAG CAG TCA GAT ATG AAC G-3′, and the antisense primer 5′-CTA CTG TCC CAT ATC CAG GTG C-3′. PCR was performed for 28 cycles , with each cycle consisting of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, followed by a final extension step at 72 °C for 8 min. Amplified PCR products were subjected to 2% agarose gel electrophoresis, and were stained using ethidium bromide for detection.

Antibody preparation and Western blotting

Glutathione S-transferase (GST) fusion proteins of full-length ARIP2b (amino acids 1–101) were used for immunization of New Zealand White rabbits. To obtain polyclonal antibodies recognizing the COOH-terminal region of ARIP2b/2c, but not ARIP2 and other PDZ proteins, crude rabbit antibodies were purified using protein A-Sepharose 4B (Amersham Biosciences), and affinity-chromatography of GST fusion proteins with N-terminal amino acids 1–82 of ARIP2b. The antibodies that recognize the COOH-terminal region of ARIP2b/2c, but not ARIP2, were thus obtained and used for Western blot analysis. Multiple mouse tissues were homogenized in buffer (1% Triton X-100, 50 mM Tris–HCl (pH 7·5), 150 mM NaCl, 1 mM PMSF, 4 μg/ml leupeptin, 1 μg/ml aprotinin), and centrifuged to yield supernatant fractions. Proteins were immunoprecipitated by adding an anti-ARIP2b/2c polyclonal antibody to the supernatant, and then incubated with protein G-sepharose (Amersham Pharmacia Biotech) at 4 °C for 2 h. Precipitated proteins were fractionated by SDS-PAGE using a 15% gel, and transferred onto a PVDF membrane. Membranes were initially incubated with an anti-ARIP2b/2c polyclonal antibody (diluted 1:500), and then with a horseradish peroxidase-conjugated secondary antibody. As a loading control, membranes were also probed with anti-actin antibody (Sigma).

Activin-responsive promoter analysis

The CAGA-lux construct has been described previously (Dennler *et al.* 1998). CAGA-lux, CMV-β-gal, pCIneo-ARIP2b and pcDNA3-ARIP2c were introduced into HEK 293 cells or LβT2 cells using TransFast liposome reagents (Promega) in accordance with the manufacturer's protocol. Stimulation by activin A and measurement of luciferase activity were performed as described previously (Tsuchida *et al.* 1995). We used activin A from WAKO Chemicals (Osaka, Japan) that is fully active.

Radioimmunoassay for follicle stimulating hormone (FSH)

LβT2 cells were grown in 12-well plates in DMEM supplemented with 10% fetal calf serum (FCS). ARIP2b cDNA was introduced into LβT2 cells using TransFast liposome reagents. Stimulation by 50 ng/ml activin A and measurement of FSH levels in conditioned media by radioimmunoassay (RIA) were performed as previously described (Graham *et al.* 1999). The sensitivity of the RIA was 4 ng/ml.

Cell surface ^{125}I-activin binding and internalization assay

CHO-K1 cells grown in 6-well plates were transfected with ActRIIA cDNA with or without pcDNA3-ARIP2c using TransFast liposome reagents. To examine total (cell surface and internalized) activin binding activity of cells, transfected cells were washed three times with cold binding medium (minimum essential medium containing 20 mM HEPES-NaOH (pH 7·4) and 0·1% bovine serum albumin), and incubated in 800 μl cold binding medium containing 15 ng ^{125}I-activin A labeled by the chloramine T method (Matsuzaki *et al.* 2002) for the indicated time periods on ice. Then unbound ligands were removed by washing three times with cold PBS. Cells were solubilized in 1 N NaOH, and the radioactivities of the cells were counted in a γ counter. To study internalization of ActRIIA, transfected cells were washed three times with binding medium, and incubated in 800 μl binding medium containing 15 ng ^{125}I-activin A at 37 °C for the

appropriate time periods. Following incubation, plates were placed on ice, washed three times with cold PBS, and then incubated with cold acid washing buffer (0·2 M acetic acid and 0·5 M NaCl) for 5 min on ice. Then, cells were solubilized in 1 M NaOH, and the radioactivities in the lysates were counted as above. Cell-associated radioactivity after the acid wash represented internalized activin mainly through receptor binding. Internalization was determined as the ratio of internalized ^{125}I-activin-A/surface bound ^{125}I-activin-A. Nonspecific binding and internalization activities were determined in the presence of a 100-fold excess of unlabeled activin A.

Determination of ActRIIA expression at the cell surface

HEK 293 cells in 6-well dishes were transfected with myc-tagged ActRIIA cDNA and/or pcDNA3-ARIP2c expression plasmids, then cells were harvested in 200 µl PBS at various time points after transfection. Cells were then treated with 3 µl rabbit anti-myc antibody at 4 °C for 2 h to label ActRIIA at the cell surface, washed three times with PBS, and then extracted with lysis buffer as described in the Materials amd Methods section. Labeled receptors were collected with protein A sepharose, and detected by blotting with anti-ActRIIA antibody as described above.

Quantitative measurement of cell surface ActRIIA associated with ARIP2

HEK 293 cells were co-transfected with myc-tagged ActRIIA, FLAG-ARIP2 and/or FLAG-ARIP2c cDNAs. To quantitate ARIP2s that associate with ActRIIA, cell proteins were extracted with lysis buffer and immunoprecipitated with anti-myc antibody, and co-precipitated ARIP2s were detected by blotting with anti-FLAG antibody. To detect cell surface ActRIIA associated with ARIP2s, myc-tagged ActRIIA at the cell surface was labeled with myc antibody, collected by protein A sepharose and blotted with anti-FLAG antibody as described above.

Detection of recycling ActRIIA associated with ARIP2

HEK 293 cells were transfected with myc-tagged ActRIIA, FLAG-ARIP2 and FLAG-ARIP2c cDNAs. Twenty-four hours after transfection, cells were washed with PBS and exposed to 0·025% trypsin for 10 min at 4 °C. At the end of trypsin treatment, cells were washed three times with medium containing 10% FCS to stop trypsin activity. Then, cells were re-incubated in medium with 10% FCS at 37 °C, and treated either with 500 mM chloroquine or 50 mM monensin from WAKO Chemicals. Cells were collected at appropriate times. Cell surface myc-ActRIIA was labeled with myc antibody, collected with protein A sepharose and blotted with anti-FLAG antibody to detect ARIP2s as described above. To detect recycling ActRIIA associated with ARIP2s, total lysate was immunoprecipitated with myc antibody, then co-precipitated ARIP2s were detected by blotting with anti-FLAG antibody.

Results

Identification of isoforms of ARIP2

In the search for intracellular proteins that interact with ActRIIs, we obtained about 30 positive clones, including ARIP1 and ARIP2, with the yeast two-hybrid screening. Association of ARIP1 and ARIP2 with ActRIIA in mammalian cells was verified by co-immunoprecipitation and mammalian two-hybrid analysis (Shoji *et al.* 2000, Matsuzaki *et al.* 2002). Screening of a mouse brain cDNA library with the 462 bp coding region of ARIP2 as hybridization probe yielded full length cDNA clones of ARIP2b and 2c, and ARIP2 (Fig. 1A). ARIP2 encoded a protein of 153 amino acids including the single PDZ domain as reported previously (Matsuzaki *et al.* 2002). ARIP2b cDNA encoded a protein of 101 amino acids with one PDZ domain which was identical to that of ARIP2 except for 8 amino acids at the NH_2-terminal. However, the ARIP2b amino acid sequences differed from ARIP2 outside the PDZ domain (Fig. 1A,B). ARIP2c is composed of 118 amino acids, and is completely identical to ARIP2 from the NH_2-terminal region through the PDZ domain, but its COOH-terminal region is identical with that of ARIP2b (Fig. 1A).

ARIP2b and 2c interact specifically with ActRIIA among serine/threonine kinase receptors

PDZ domains recognize a class I consensus PDZ-binding motif XSX(V/I/L) (where X is any amino acid) in the COOH terminus of target proteins (Songyang *et al.* 1997). In agreement with the consensus PDZ-binding sequence, the four COOH-terminal amino acids of ActRIIA were ESSL. ARIP2b interacted with ActRIIA, but did not associate with ActRIIB, TGF-β receptor type II (TGFβRII) or BMP receptor type II (BMPRII) by mammalian two hybrid analysis (Fig. 2A). Like ARIP2b, ARIP2c also interacted with ActRIIA. ARIP2c did not interact with mutated ActRIIA proteins that lacked either the COOH-terminal SSL or COOH-terminal leucine (Fig. 2B). Similar results were obtained for ARIP2b (data not shown). Thus, COOH-terminal amino acids of ActRIIA play a key role in interactions between ARIP2b and ARIP2c via the PDZ domain. Amino acid sequences of target proteins relate to specific interactions with various PDZ proteins. It is worthwhile noting that although ARIP2 interacted with both ActRIIA and ActRIIB, ARIP2b and 2c interacted with ActRIIA,

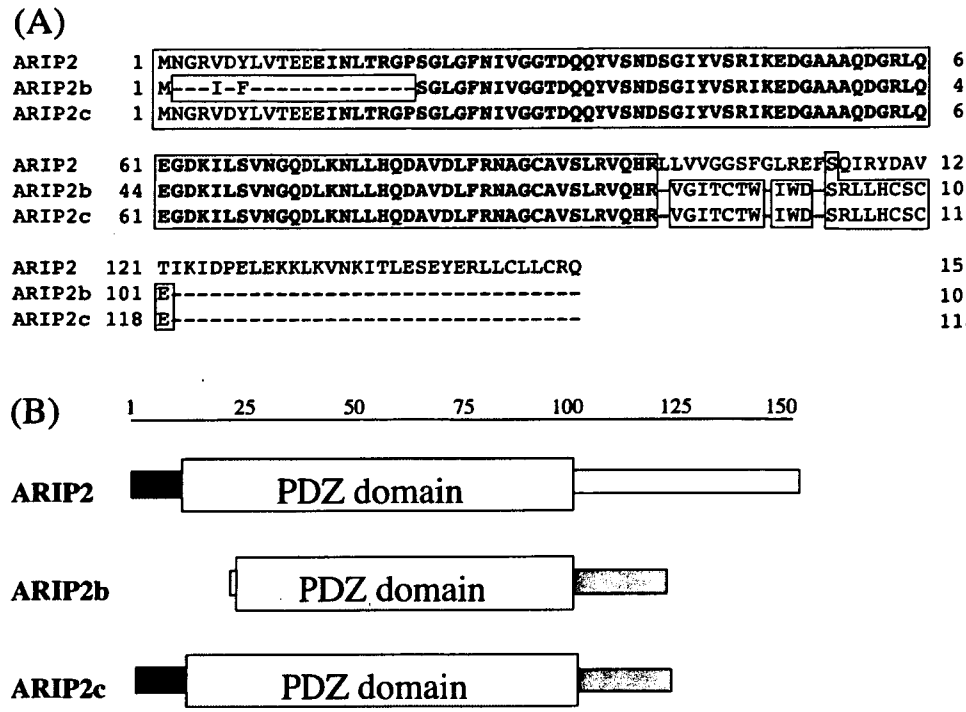


Figure 1 Identification of ARIP2 isoforms. (A) Alignment of amino acid sequences of ARIP2 and its two isoforms, ARIP2b and ARIP2c. PDZ domains are indicated in bold letters. Identical amino acids are boxed. (B) Schematic illustration of protein structures of ARIP2 along with those of ARIP2b and 2c. The NH_2-terminal amino acid residues in ARIP2b that differ from ARIP2 are shown as a white box. The COOH-terminal amino acid residues unique in ARIP2 are indicated as a white box. The COOH-terminal amino acid residues in ARIP2b/2c that differ from ARIP2 are shown as gray boxes.

but not with ActRIIB (Fig. 2A,B). These results indicate that ARIP2b or 2c interact specifically with ActRIIA among type II serine/threonine kinase receptors of the TGF-β superfamily, and that this interaction occurs in both yeast and mammalian cells. We then studied which part of ARIP2c interacted with ActRIIA using a mammalian two-hybrid method. ARIP2cΔC, but not ARIP2cΔPDZ interacted with ActRIIA, indicating that ARIP2c interacts with ActRIIA through a PDZ domain-mediated interaction (Fig. 2C). ARIP2b, 2c and ARIP2 have an identical amino acid sequence in the PDZ domain which is important for interactions with ActRIIA. Lysates from COS-7 cells that have been co-transfected with ActRIIA, and FLAG-tagged ARIP2c were incubated with an anti-ActRIIA antibody, and co-immunoprecipitated ARIP2c protein was detected by an anti-FLAG antibody. As shown in Fig. 2D, ActRIIA and ARIP2c formed a protein complex in transfected cells. Similar interactions of ActRIIA and ARIP2b were observed (data not shown). ARIP1 interacts with Smads via WW domains, whereas ARIP2 does not interact with Smad proteins (Shoji *et al.* 2000, Matsuzaki *et al.* 2002). ARIP2 isoforms, i.e. ARIP2b and 2c, like ARIP2, did not show any interaction with Smad (data not shown).

Expression of ARIP2 isoforms in mouse tissues

Northern blot analysis of poly(A)$^+$RNA extracted from several mouse tissues using full length ARIP2 cDNA probe yielded two major bands of 4·5 kb and 1·1 kb (Matsuzaki *et al.* 2002). Since very short sequences in the COOH-terminal of ARIP2b/2c are different from ARIP2, and ARIP2 has an additional isoform called synaptojanin binding protein (also known as outer membrane protein of 25 kDa, OMP25) (Nemoto & DeCamilli 1999), it is difficult to detect ARIP2b/2c by Northern blotting. Therefore, we compared the tissue distribution of ARIP2 and ARIP2c mRNAs using RT-PCR. As shown in Fig. 3A, both ARIP2 and ARIP2c are widely expressed in various mouse tissues. ARIP2 mRNA was detected in multiple tissues. ARIP2c mRNA was detected in brain, liver, kidney, ovary and testis Although ARIP2 and 2c are structurally similar, their distributions differ slightly. ARIP2b mRNA was detected in heart, spleen and testes by RT-PCR, but its expression levels were much lower than those of ARIP2c mRNA (data not shown). We also studied protein expression of ARIP2b/2c. Antibodies that recognized ARIP2b/2c but not ARIP2 or OMP25 were prepared and used for Western

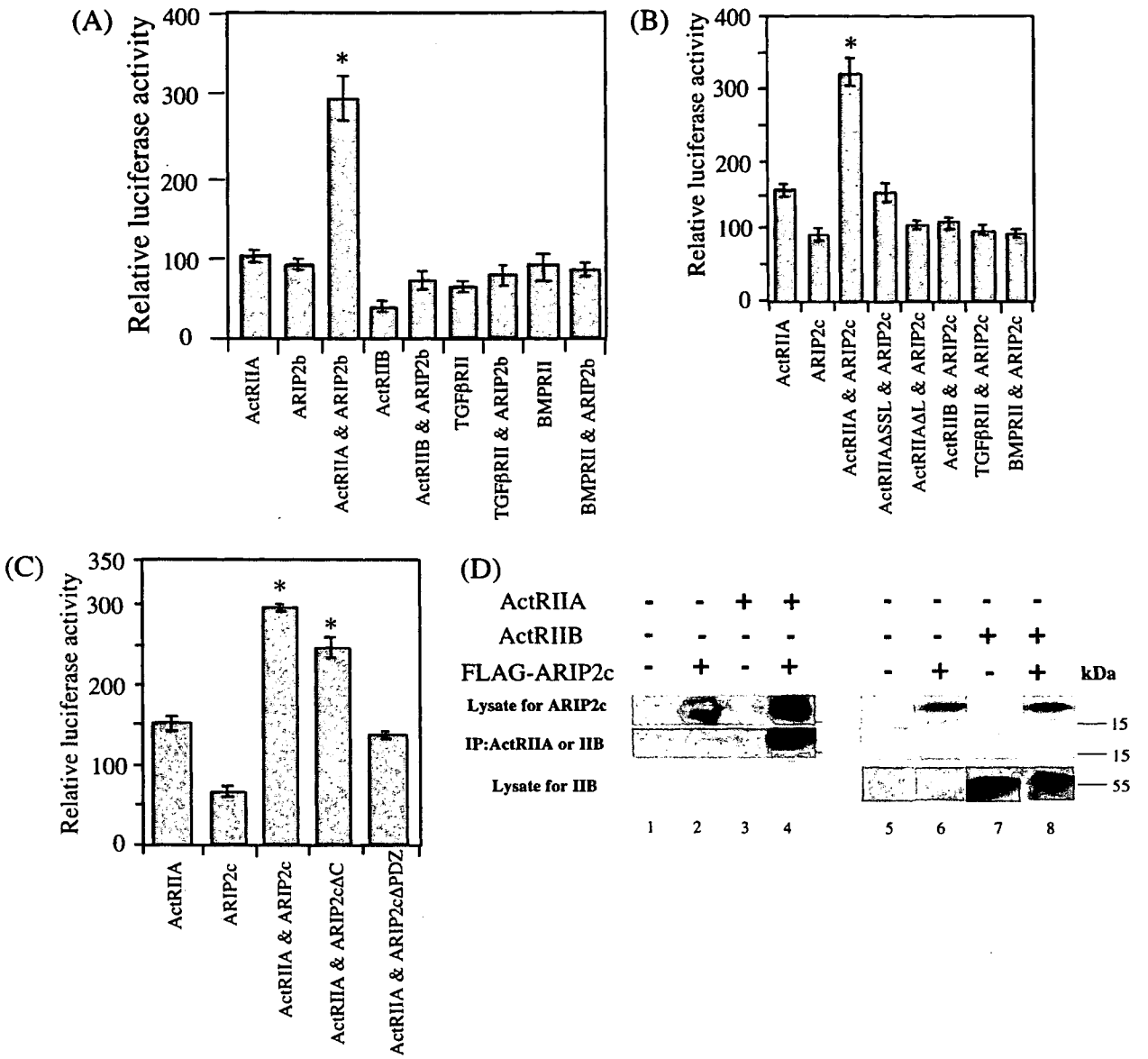


Figure 2 Interactions of ARIP2b or 2c with ActRIIA assessed by mammalian two-hybrid analysis. (A) Interaction of ARIP2b with ActRIIA, ActRIIB, TGFβRII and BMPRII in CHO-K1 cells. Relative interaction is shown as luciferase activity. Expression vectors used were pACT-ARIP2b and pBIND plasmids, into which the entire cytoplasmic region of each receptor was introduced. Values in the figure represent means and S.D. of triplicate determinations. *$P<0{\cdot}01$ vs ActRIIA using a *t*-test. (B) Interaction of ARIP2c with ActRIIA, ActRIIAΔSSL, ActRIIAΔL, ActRIIB, TGFβRII and BMPRII in CHO-K1 cells. Relative interaction is represented as luciferase activity. *$P<0{\cdot}01$ vs ActRIIA using a *t*-test. (C) Interaction of ARIP2c, ARIP2cΔC or ARIP2cΔPDZ with ActRIIA in CHO-K1 cells. Relative interaction is represented as luciferase activity. Values in the figure represent means and S.D. of triplicate determinations. *$P<0{\cdot}01$ vs ActRIIA using a *t*-test. (D) Interaction of ActRIIA with ARIP2c by immunoprecipitation (IP). In lanes 1–4, lysates of COS-7 cells transfected with either pcDNA3-FLAG-ARIP2c (lane 2), or pcDLSRα-ActRIIA (lane 3), or both (lane 4), or untransfected (lane 1) were immunoprecipitated with an anti-ActRIIA antibody, and probed with an anti-FLAG monoclonal antibody (bottom row). On the top row, transfected COS-7 cells were probed with an anti-FLAG antibody. The molecular weight marker is indicated on the right. In lanes 5–8, COS-7 cells transfected either with pcDNA3-FLAG-ARIP2c (lane 6) or pcDLSRα-ActRIIB (lane 7) or both (lane 8), or untransfected (lane 5) were immunoprecipitated with an anti-ActRIIB antibody, and probed with an anti-FLAG monoclonal antibody (middle row). To show expression of ActRIIB, lysates were probed with ActRIIB antibody in the bottom row.

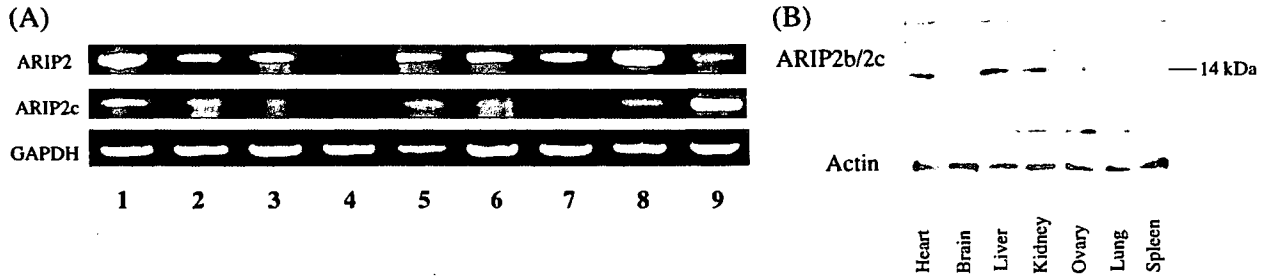


Figure 3 Distribution of ARIP2 isoforms in mouse tissues. (A) Tissue distribution of ARIP2 and ARIP2c mRNAs. mRNA isolated from various mouse tissues were identified by RT-PCR analysis. For control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also amplified. Lane 1, brain; lane 2, heart; lane 3, lung; lane 4, spleen; lane 5, liver; lane 6, kidney; lane 7, skeletal muscle; lane 8, ovary; lane 9, testis. (B) Tissue distribution of ARIP2b/2c by Western blot analysis. A polyclonal antibody recognizing the COOH-terminus of ARIP2b/2c was used for detection (top row). For control, antibody to actin was used (bottom row). The molecular weight marker is indicated on the right.

blot analysis. As shown in Fig. 3B, ARIP2b/2c signal was present in heart, liver and kidneys, and to a lesser extent in brain, ovaries and lungs.

ARIP2b/2c has a stimulatory effect on activin-induced signaling and secretion of FSH from LβT2 cells

HEK 293 cells were co-transfected with ARIP2c cDNA and the reporter plasmid CAGA-lux, and activin-induced luciferase activity was measured. CAGA-lux is a reporter plasmid that has CAGA tandem repeats, and responds well to activin and TGF-β stimuli (Dennler *et al.* 1998). Overexpression of ARIP2c augmented activin-induced transcriptional activity in a dose-dependent manner (Fig. 4A). We also studied the effects of ARIP2b on FSH secretion in gonadotroph LβT2 cells, which secrete FSH in response to activin (Graham *et al.* 1999). LβT2 cells were transfected with ARIP2b and CAGA-lux DNAs, and activin-induced luciferase activity was measured. Overexpression of ARIP2b cDNA increased activity of CAGA promoter in a dose-dependent manner (Fig. 4B). FSH secretion by activin in the culture media was actually induced by ARIP2b cDNA transfection in LβT2 cells (Fig. 4C). ARIP2c showed a similar activity to ARIP2b.

Antagonism of ARIP2 and ARIP2b on activin signaling

In a previous report, we showed that ARIP2 inhibited activin signaling (Matsuzaki *et al.* 2002). Since ARIP2 and ARIP2b/2c have opposite effects on activin signaling, we investigated whether ARIP2 and ARIP2b have antagonistic activities when expressed simultaneously. Expression of ARIP2b cDNA augmented activin signaling in a dose-dependent manner in HEK 293 cells (Fig. 4D). In the presence of ARIP2, activin signaling was inhibited. When ARIP2b was co-expressed with ARIP2, the inhibitory effect of ARIP2 was blocked by ARIP2b cDNA in a dose-dependent manner. This result indicates that ARIP2 and ARIP2b have antagonistic activities on activin signaling. ARIP2c, like ARIP2b, showed antagonistic activities to ARIP2 (data not shown). Since ARIP2b and 2c are expressed in numerous tissues, they could play a role in controlling activin signaling transduction.

ARIP2b/2c neither affects endocytosis of ActRIIA nor interacts with RalBP1

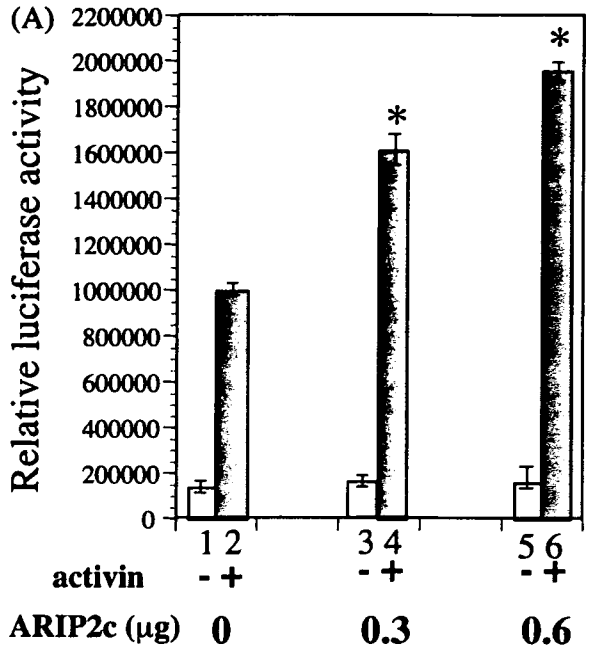
In a previous study, we reported that ARIP2 regulated endocytosis of ActRIIA through the Ral/RalBP1-dependent pathway (Matsuzaki *et al.* 2002). To study the role of ARIP2b/2c in internalization of activin through ActRIIA, we performed an internalization experiment. Unlike ARIP2, overexpression of ARIP2c did not affect internalization of ActRIIA (Fig. 5A). In a parallel experiment, we reproduced enhanced internalization of ActRIIA by ARIP2 expression. To examine interactions of ARIP2c with RalBP1, we performed an immunoprecipitation experiment. As shown in Fig. 5B, ARIP2c did not significantly interact with RalBP1, whereas ARIP2 interacted with RalBP1. ARIP2b, like ARIP2c, did not intereact with RalBP1 (data not shown).

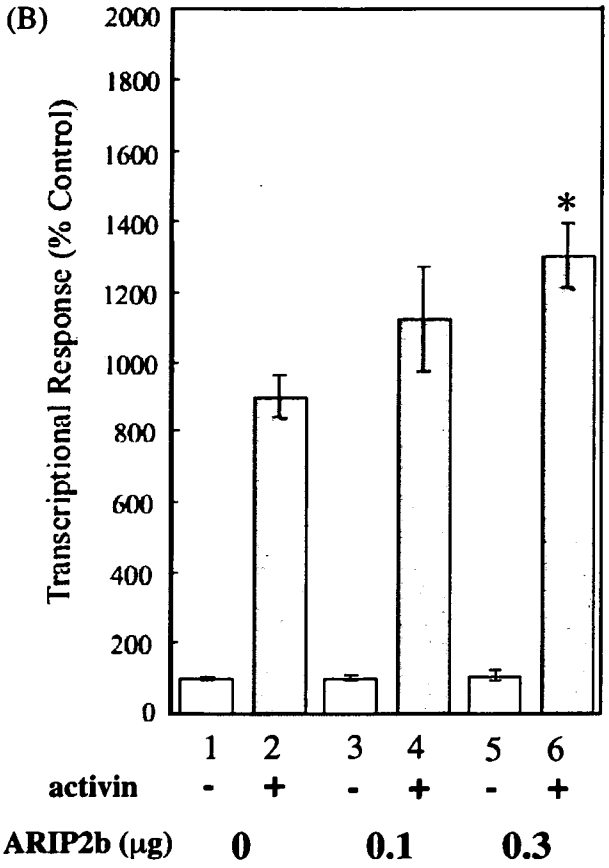
ARIP2b/2c increases expression of ActRIIA on cell surfaces

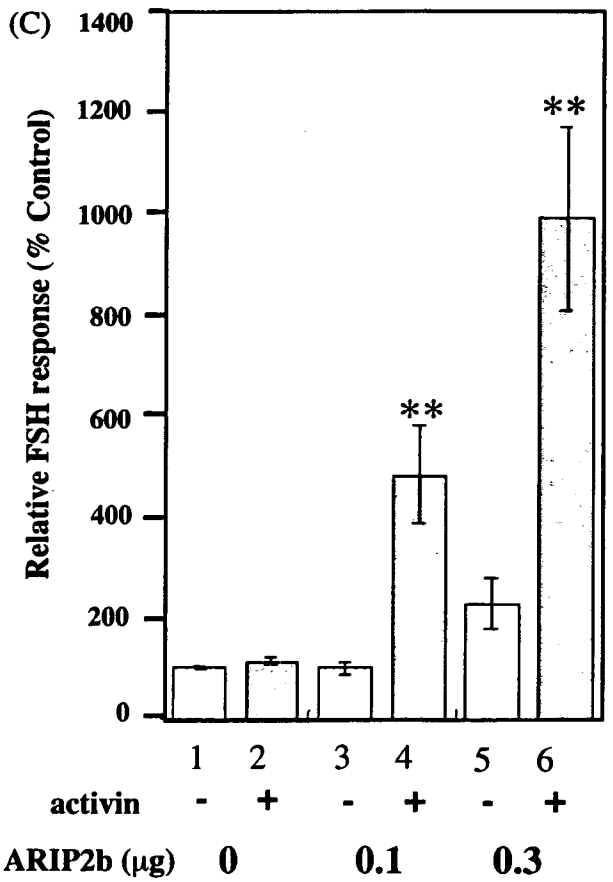
To further investigate interactions between ARIP2c and ActRIIA, we co-expressed these proteins and examined the effects on surface receptor expression levels. Figure 6A shows the effects of activity of ARIP2c on the expression of ActRIIA in HEK 293 cells. In the presence of ARIP2c, the number of ActRIIA at the cell surface increased faster after the 18 h time point compared with control (Fig. 6A). In contrast, in the presence of ARIP2, cell surface levels of ActRIIA did not significantly change when compared with control (Fig. 6B).

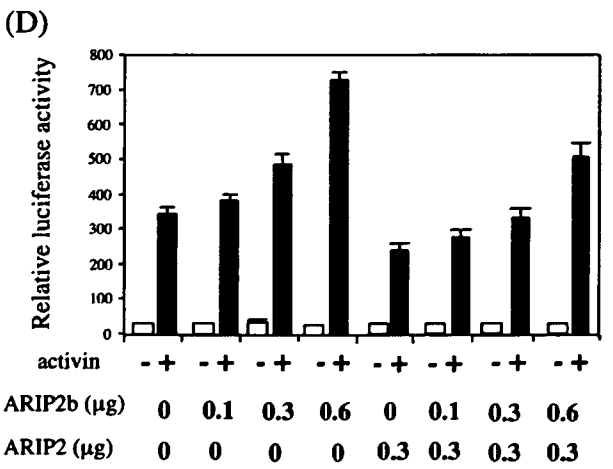
Mode of association of ARIP2c with ActRIIA is different from that of ARIP2 with ActRIIA

We next quantitated the amounts of ARIP2c and ARIP2 that associated with cell surface ActRIIA. After 6 h


(A)
Relative luciferase activity
2200000
2000000
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
*
*
1 2 3 4 5 6
activin - + - + - +
ARIP2c (μg) 0 0.3 0.6


(B)
Transcriptional Response (% Control)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
1 2 3 4 5 6
activin - + - + - +
ARIP2b (μg) 0 0.1 0.3


(C)
Relative FSH response (% Control)
1400
1200
1000
800
600
400
200
0
**
**
1 2 3 4 5 6
activin - + - + - +
ARIP2b (μg) 0 0.1 0.3


(D)
Relative luciferase activity
800
700
600
500
400
300
200
100
0
activin - + - + - + - + - + - + - + - +
ARIP2b (μg) 0 0.1 0.3 0.6 0 0.1 0.3 0.6
ARIP2 (μg) 0 0 0 0 0.3 0.3 0.3 0.3

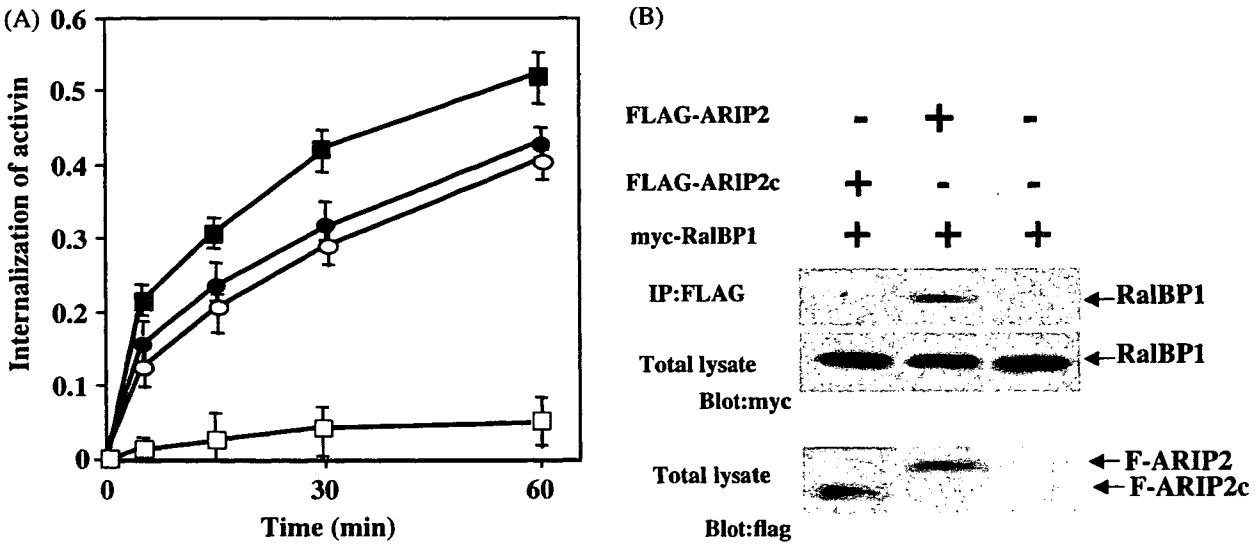


Figure 5 Effects of ARIP2c on ActRIIA internalization. (A) CHO-K1 cells were transfected with control vector (□), ActRIIA (○), ActRIIA and ARIP2c (●) or ActRIIA and ARIP2 (■), treated in binding buffer with 15 ng ^{125}I-activin A, and incubated at 37 °C for appropriate time periods. Graphs show internalization of ActRIIA calculated using the ratio of internalized activin/surface activin binding as described in Materials and Methods. Values shown are means and S.D. of triplicate determinations. (B) Interaction of RalBP1 with ARIP2 or ARIP2c. COS-7 cells were transfected with myc-RalBP1 and FLAG-ARIP2 or FLAG-ARIP2c, and then lysates were immunoprecipitated (IP) with anti-FLAG antibody, separated by SDS-PAGE, blotted onto a membrane, and probed with anti-myc antibody. In the middle row, total lysate was analyzed for RalBP1 expression. In the bottom row, total lysate was analyzed for FLAG (F)-ARIP2 or F-ARIP2c expression.

transfection, ARIP2 and ARIP2c began to be expressed, then increased and reached a maximal value at 18 h, and finally remained constant from 18 to 36 h after transfection (Fig. 6C, middle row). After 12 h transfection, ARIP2c co-precipitated with cell surface ActRIIA, and gradually reached a maximal amount from 18 to 36 h. In contrast, ARIP2 co-precipitated with cell surface ActRIIA at a much slower rate than ARIP2c. After 18 h transfection, co-precipitation of ARIP2 was observed, and slowly increased thereafter (Fig. 6C, top row). We also investigated total (cell surface and intracellular) ActRIIA associating with ARIP2 or ARIP2c (Fig. 6D). ARIP2c co-precipitated with total ActRIIA after 6 h transfection, then increased to reach a maximal amount and then remained constant from 18 to 36 h. In contrast, ARIP2 co-precipitated with ActRIIA after 12 h transfection, then slowly increased. We studied differences in association mechanisms of ARIP2 and ARIP2c to cell surface ActRIIA. First, we trypsinized transfected HEK 293 cells to degrade cell surface receptor binding activities, and newly synthesized ActRIIA associating with ARIP2s was quantitated at various time points by co-immunoprecipitation. As shown in Fig. 7A, after 2 h trypsin stripping, cell surface receptor-associated ARIP2 and ARIP2c were observed, and increased rapidly. Almost the same amounts of ARIP2 and ARIP2c co-precipitated with cell surface ActRIIA (Fig. 7A). Next, we used cycloheximide to block new protein synthesis after trypsin treatment, and measured the amounts of ARIP2 and ARIP2c that bound to newly appearing ActRIIA at the

Figure 4 Effects of ARIP2b or 2c on activin-induced transcription. (A) ARIP2c-mediated increase in activin-induced transcription in HEK 293 cells. Cells were transfected with CAGA-lux, CMV-β-gal, and various amounts of ARIP2c cDNA, and then incubated with 50 ng/ml activin A for 12 h. The level of luciferase activity of each cell lysate was measured, and normalized against the level of β-gal activity. Values in the figure represent means and S.D. of triplicate determinations. *P<0·01 vs activin-treated cells without ARIP2c (lane 2) using a t-test. (B) Effects of ARIP2b on activin-induced transcription in LβT2 cells. Cells were transfected with CAGA-lux, CMV-β-gal, and various amounts of ARIP2b cDNA, and then incubated with 50 ng/ml activin A for 12 h. The level of luciferase activity of each cell lysate was measured, and normalized against the level of β-gal activity. Values in the figure represent means and S.D. of triplicate determinations. *P<0·05 vs activin-treated cells without ARIP2b (lane 2) using a t-test. (C) Activin-mediated increase in FSH secretion from LβT2 cells. Cells were transfected with various amounts of ARIP2b cDNA, and incubated with 50 ng/ml activin A for 24 h. FSH was assayed by a radioimmunoassay kit from NIDDK (Bethesda, MD, USA). FSH secretion from untransfected cells was 5·0 ng/ml, and was adjusted to 100. ** P<0·01 vs activin-treated cells without ARIP2b (lane 2) using a t-test. (D) Antagonist effects of ARIP2b and ARIP2 on activin signaling. HEK 293 cells were transfected with CAGA-lux, CMV-β-gal, and various amounts of ARIP2 and/or ARIP2b cDNA, and then incubated with 50 ng/ml activin A for 12 h. The level of luciferase activity of each cell lysate was measured and normalized against the level of β-gal activity. Values in the figure represent means and S.D. of triplicate determinations.

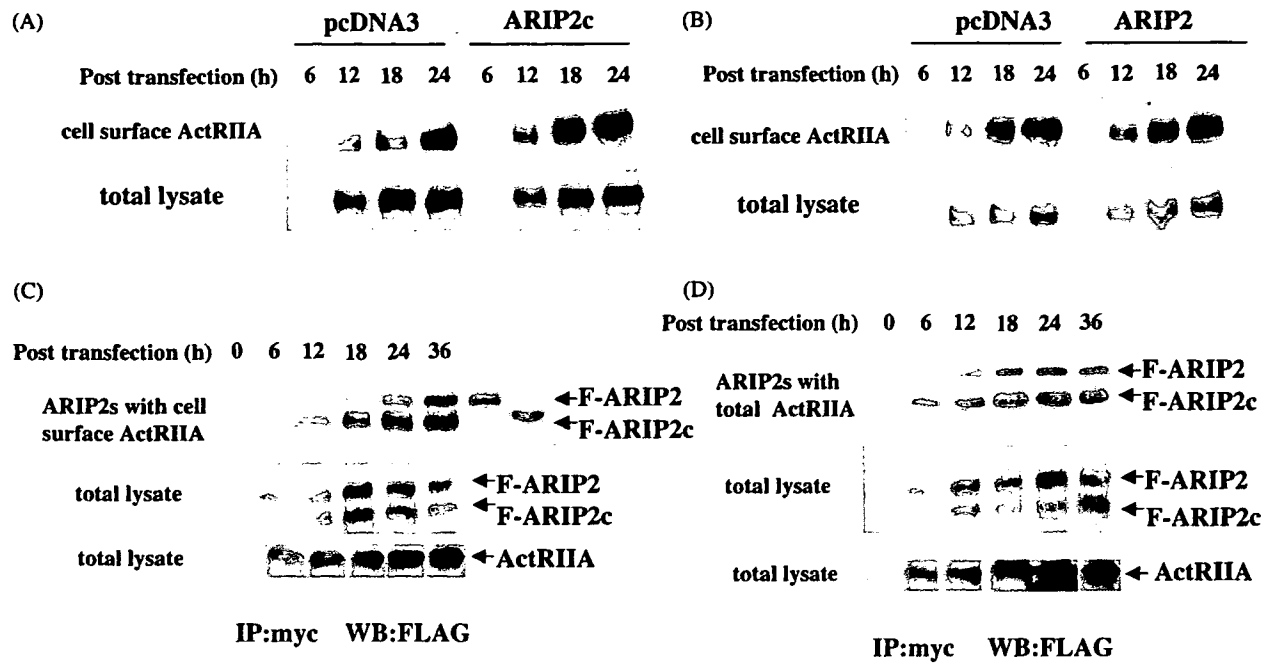


Figure 6 Effects of ARIP2c on cell surface level of ActRIIA and association of ARIP2/ARIP2c with ActRIIA. (A) ARIP2c increases expression of ActRIIA at the cell surface. HEK 293 cells were transfected either with myc-tagged ActRIIA (left panel) or myc-tagged ActRIIA and ARIP2c cDNAs (right panel). After appropriate time periods, cells were harvested and treated with anti-myc antibody to label cell surface receptors, immunoprecipitated and detected by blotting with anti-ActRIIA antibody. (B) Same experiment as in (A) except that ARIP2 cDNA was used instead of ARIP2c cDNA. (C) HEK 293 cells were transfected with myc-tagged ActRIIA, FLAG (F)-ARIP2 and F-ARIP2c cDNAs. Then cell surface receptors were labeled with myc antibody, extracted, immunoprecipitated (IP), and blotted with FLAG antibody. In the middle and bottom rows, total lysate was analyzed. In the two rightmost lanes in the top row, positions of ARIP2 and ARIP2c are shown by comparison. (D) Same experiment as in (C) except that cell surface labeling with myc antibody was omitted to detect ARIP2s associating with total (cell surface and intracellular) ActRIIA. In the middle and bottom rows, total lysate was analyzed. WB, Western blotting.

plasma membrane (Fig. 7B). Although almost equal amounts of ARIP2 and ARIP2c were observed in the total lysate, only small amounts of ARIP2c that co-precipitated with cell surface ActRIIA were detected (Fig. 7B). This result indicates that ARIP2c, possibly acting in a dominant-negative manner to block ARIP2, is involved in blocking endocytosis of ActRIIA by ARIP2. Then, we studied the association of ARIP2 or ARIP2c with ActRIIA using the recycling inhibitors, chloroquine or monensin. When chloroquine was used to interfere with recycling after trypsin treatment, the amounts of cell surface ARIP2 associating with ActRIIA significantly decreased, whereas the presence of chloroquine did not significantly affect the amounts of ARIP2c co-precipitating with cell surface ActRIIA (Fig. 7C, left panel). When monensin was used to prevent recycling of the internalized receptor back to the cell surface, only a small amount of ARIP2 co-precipitated with cell surface ActRIIA (Fig. 7C, right panel). Even without trypsin treatment, chroloquine treatment affected the amounts of cell surface ActRIIA associated with ARIP2, whereas it did not significantly affect the amounts of ARIP2c co-precipitating with cell surface ActRIIA (Fig. 7D). Taken together, these results indicate that ARIP2 is involved in endocytosis and recycling of ActRIIA, whereas ARIP2c, acting in a dominant-negative manner to block ARIP2, enhances ActRII levels at the cell surface.

Discussion

Activin type II receptors have been identified in multiple tissues, and are involved in signaling pathways of activins (Sugino & Tsuchida 2000, Tsuchida 2004). Activins first bind to type II receptors, then type II receptors induce phosphorylation and activation of type I receptors. Then, type I receptors activate Smads to propagate signal transduction. ActRIIs retain a basal level of autophosphorylation that is independent of either type I receptors or ligand binding. Furthermore, type II receptors undergo endocytosis by adaptor proteins (Matsuzaki *et al.* 2002, Di Guglielmo *et al.* 2003). These findings suggest that type II receptors act as primary signal determinants. As a consequence, factors that control the actions of type II receptors regulate downstream propagation of activin-induced signaling. In the present study, we isolated and characterized

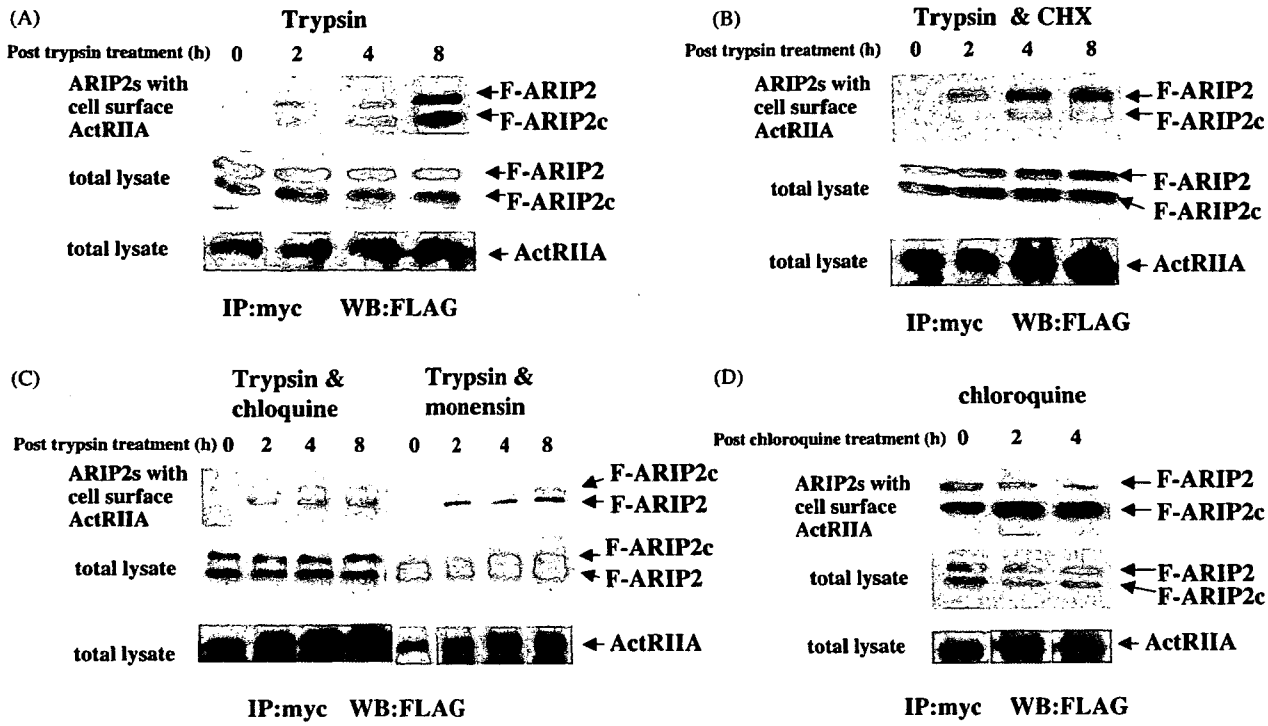


Figure 7 ARIP2 is involved in endocytosis of ActRIIA, whereas ARIP2c regulates surface expression of ActRIIA. (A) HEK 293 cells were transfected with myc-tagged ActRIIA, FLAG (F)-ARIP2 and F-ARIP2c cDNAs. After 24 h, cells were treated with trypsin, washed and then reincubated for appropriate time periods. Then, cell surface receptors were labeled with myc antibody, extracted, immunoprecipitated (IP) and blotted with FLAG antibody. In the middle and bottom rows, total lysate was analyzed. (B) Same experiment as in (A) except that cells were incubated in the presence of cycloheximide (CHX) for appropriate time periods after trypsin treatment to inhibit protein synthesis. (C) Same experiment as in (A) except that cells were incubated in the presence of chloroquine (left panel) or monensin (right panel) for appropriate time periods after trypsin treatment to inhibit recycling. (D) Same experiment as in left panel of (C) except that trypsin treatment was omitted. WB, Western blotting.

two isoforms of ARIP2, named ARIP2b and ARIP2c. Interestingly, unlike ARIP2, overexpression of ARIP2b or 2c enhanced activin signaling. ARIP1 and ARIP2 also interacted with ActRIIs, but they inhibited activin signal transduction when overexpressed (Shoji *et al.* 2000, Matsuzaki *et al.* 2002). ARIP2b and ARIP2c are unique in that they associate with ActRIIA and enhance activin signaling. In a previous study, we reported characterization of a PDZ domain deletion mutant of ARIP2 called ARIP2ΔC. Interestingly, ARIP2ΔC, like ARIP2b and 2c, enhances activin signaling (Matsuzaki *et al.* 2002). We did not detect significant interaction between ARIP2b/2c and ActRIIB. Similarly, there was no significant interaction between ARIP2b/2c and either TGFβRII or BMPRII (Fig. 2A). Analysis of interactions between ARIP2b/2c and ActRIIAs showed that ARIP2b/2c interact with the COOH terminus of ActRIIAs through the PDZ domain. The PDZ domains of ARIP2 and its isoforms are identical. The three COOH terminal amino acids of ActRIIA, consistent with the PDZ-binding motif, are critical for this interaction (Fig. 2). ARIP2 interacts with ActRIIA via its PDZ domain and with RalBP1 via its COOH-terminus, resulting in both endocytosis of activin type II receptors and an attenuated activin-induced transcriptional response (Matsuzaki *et al.* 2002). ARIP2b and 2c did not interact with RalBP1. Overexpression of ARIP2b or 2c augmented activin-induced transcription in HEK 293 cells (Fig. 4A), and increased activin-induced secretion of FSH from LβT2 cells (Fig. 4C). Further characterization revealed that, unlike ARIP2, ARIP2b/2c did not affect internalization of ActRII and showed antagonistic activity to ARIP2. Whether ARIP2b or 2c increase signal transduction in physiological conditions remains to be determined. Since ARIP2 and ARIP2b/2c have opposite effects on receptor localization and function, the ratio of ARIP2 to its isoforms, ARIP2b/2c, likely regulates ActRIIA activity. Our characterization of ARIP2 and its isoforms offers another example of isoforms having different functions.

Involvement of Smad proteins in cellular signaling induced by the TGF-β superfamily has been extensively studied (Heldin *et al.* 1997). In addition, there is accumulating evidence that multiple adaptors and scaffolding proteins interact with receptor serine/threonine kinases.

ARIP1 and ARIP2 interact with ActRIIA and ActRIIB. Dok-1 shows homologies to pleckstrin and to phosphotyrosine-binding domains that associate with activin and TGF-β receptors, and is a key element in activin-mediated apoptosis in B cells (Yamakawa *et al.* 2002). TRIP1 is a WD domain-containing protein that interacts with TGF-β type II receptors (Chen *et al.* 1995). TRAP-1-like protein (TLP) also associates with activin and TGF-β receptors (Felici *et al.* 2003). Interestingly, TLP suppresses Smad3-dependent signaling but potentiates Smad2-dependent signaling. These results indicate that adaptor proteins have a role in coordinating signal transduction by regulating protein complexes including receptor serine/threonine kinase receptors. ActRIIA is shared with activins, myostatin, nodal and BMP-7. These growth factors are known to form ligand gradients. One potential mechanism for forming morphogenetic gradients involves regulation of receptor numbers at the cell surface (McDowell & Gurdon 1999). Thus, ARIP2 and its variants may have a role in shaping morphogenetic gradients and in fine-tuning activin signaling during tissue formation (McDowell & Gurdon 1999).

Our characterization of ARIP2 and its isoforms as mediators of activin signaling has revealed a novel regulation of receptor serine/threonine kinases, and adds new insights into the mechanisms of regulation of signal transduction through ActRII by PDZ proteins.

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〔ミニレビュー〕

筋萎縮をきたす神経・筋難病の新しい治療法の開発*

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要約：骨格筋は生体で最重量の臓器であり，その働きは，中枢および末梢の神経に支配されている．筋原性変性疾患の代表である筋ジストロフィーは筋萎縮をきたし，重篤な経過をたどる疾患である．また，神経原性変性疾患である筋萎縮性硬化症は，脊髄運動ニューロンの細胞死と変性により二次的に骨格筋の萎縮を引き起こし，良好な治療法に乏しい．さらに，急速な高齢化社会を迎えており，老化や寝たきりによって生じる筋力の低下（sarcopenia）を防ぐことは社会的に重要である．遺伝子治療や細胞移植治療とともに，薬物療法として，骨格筋の増殖抑制因子マイオスタチンを分子標的とする方法が新しい治療法として有望である．本稿では，筋萎縮をきたす神経・筋難病に対する治療法の開発の現状について最近の動向と進歩について概説する．

キーワード：筋萎縮，筋ジストロフィー，筋萎縮性側索硬化症，マイオスタチン，分子治療

難治性と言われる神経疾患や筋疾患にも，近年では有望かつ画期的な治療方法の開発に長足の進歩が見られている．筋萎縮をきたす疾患としては，筋ジストロフィーが代表的疾患である．近年の分子遺伝学的な解析の進歩から，多くの責任遺伝子が同定されている．筋基底膜直下の，ジストロフィン複合体を含む細胞骨格系タンパク質分子群の遺伝子欠損が多数見られる（Durbeej and Campbell, 2002; Tsuchida, 2006）．筋ジストロフィーの治療方法の3つの大きな柱としては，(1) 遺伝子治療，(2) 細胞移植治療，(3) 薬剤治療が挙げられる．遺伝子治療は，ジストロフィンなどの欠損した分子の遺伝子を導入する治療法である．ジストロフィンを小型化したマイクロジストロフィンが開発され，アデノ随伴ウイルス（adeno-associated virus, AAV）ベクターを用いた遺伝子治療が精力的に研究されている．骨格筋に分化能を有した多種類の幹細胞が同定され理解が深まっている．それに伴い，細胞移植療法にも進歩が見られている．薬物療法については，骨格筋の幹細胞が潜在的に持つ筋分化・増殖能を活性化させる薬物療法が有望である．なかでも，マイオスタチンと呼ばれる骨格筋の量を負に制御する細胞増殖因子を分子標的とした治療方法は，最も現実的な治療法として期待されている．本ミニレビューでは，筋萎縮をきたす神経・筋難病の新しい治療法の開発とその現状について述べたい．

I. 骨格筋の発生と筋分化

骨格筋は，中胚葉組織である体節と呼ばれる分節構造に由来する．骨格筋前駆細胞が分裂を繰り返し，分化し，細胞融合によって細長い多核細胞になり，筋線維を形成する．骨格筋は，生後も高い再生能力を有しており，筋損傷時に筋再生が行われる．筋線維の周囲には，筋衛星細胞（筋サテライト細胞）と呼ばれる転写因子 Pax7 陽性の単核細胞が存在しており，筋再生を担っている．通常，筋衛星細胞は静止状態にあるが，筋損傷が起こると，活性化され，前駆細胞へ分化し分裂と増殖を繰り返した後に，融合し筋管細胞を形成する（Parker et al, 2003）．最終的に形成された骨格筋には，素早い収縮力を持つ速筋（II型筋線維）と，ミトコンドリアに富み持久力に優れた遅筋（I型筋線維）が混在し，役割分担を行っている．骨格筋と脊髄の運動ニューロンは，神経筋接合部のシナプスを介して連絡し円滑なシナプス伝達が営まれている．

II. 骨格筋の増殖と分化を支配するシグナル分子

骨格筋の形成には，細胞外から作用する細胞増殖因子や細胞接着因子と，細胞内筋分化制御因子の働きが特に重要である．1997年に，TGF-beta スーパーファミリーに属する分子である growth differentiation factor 8（GDF8）が発見された．この分子は遺伝子破壊マウスで，劇的に全身の骨格筋量が増大することから，マイオスタチン（MSTN）と呼ばれるようになった（Lee, 2004）．マイオスタチンは，胚および成体の骨格筋に主に発現し，筋線維の数や大きさを決定する重要因子である．その働きとして，産生された骨格筋周辺でのオートクリン・パラクリン作用とともに，

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略語　**AAV**: adeno-associated virus, **ALK**: activin receptor-like kinase, **ALS**: amyotrophic lateral sclerosis, **GDF8**: growth differentiation factor 8, **GLT-1**: glutamate transporter 1, **HGF**: hepatocyte growth factor, **IGF**: insulin like growth factor, **MRF**: myogenic regulatory factor, **SOD1**: Cu/Zn superoxide dismutase 1

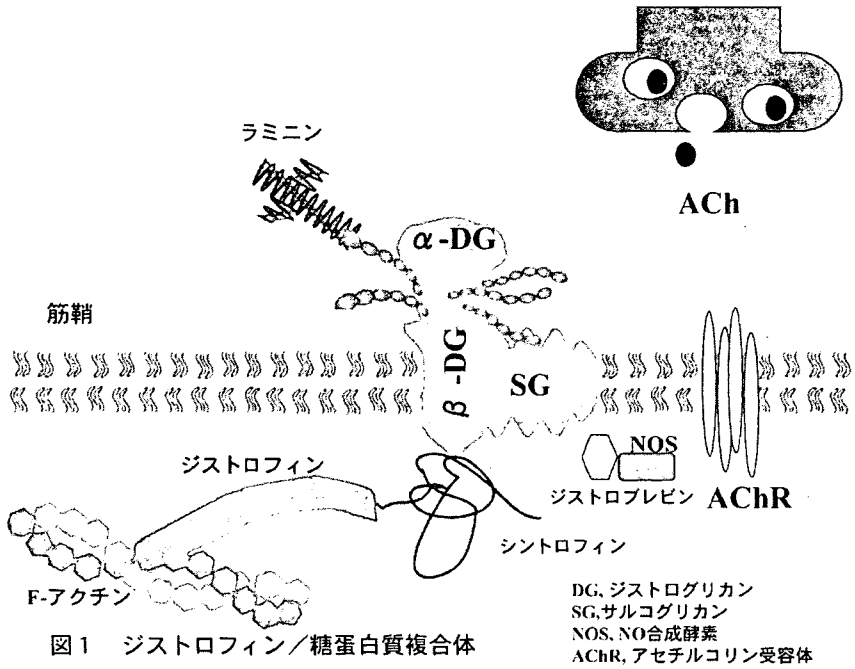

図1　ジストロフィン／糖蛋白質複合体

図1　ジストロフィン/糖蛋白質複合体.

表1　代表的な筋ジストロフィーの病型と原因遺伝子

病型	遺伝子座	原因分子
Duchenne	Xp21	Dystrophin
LGMD2D	17q12-q21	α-sarcoglycan
LGMD2E	4q12	β-sarcoglycan
LGMD2C	13q12	γ-sarcoglycan
LGMD2F	5q33-q34	δ-sarcoglycan
MDC1A	6q22	Laminin α-2
LGMD1C	3p25	Caveolin-3
LGMD2A	15q15	Calpain-3
LGMD2B	2p13	Dysferlin
Fukuyama	9q31-q33	Fukutin

LGMD: limb-girdle muscular dystrophy（肢帯型筋ジストロフィー），MDC: muscular dystrophy congenital type（先天性筋ジストロフィー）.

血液中を循環し骨格筋に働きかける内分泌作用が想定される．マイオスタチンは，骨格筋幹細胞や未分化前駆細胞に作用し，p21の誘導，MyoDの抑制を介して筋分化・増殖に対して強力な抑制作用を発揮する．マイオスタチンは細胞膜受容体をアクチビンと共有しており，II型受容体としてActRIIB，ActRIIA，I型受容体としてactivin receptor-like kinases 4, 5（ALK4, ALK5）を介して細胞内に情報を伝達する．細胞内では，Smad2, Smad3経路が情報伝達を仲介している．この点は，アクチビンやTGF-betaと類似している．一方，マイオスタチンとは逆に，筋分化を促進する因子としては，インスリン様増殖因子（insulin like growth factor, IGF），肝細胞増殖因子（hepatocyte growth factor, HGF），ガレクチン1が知られている（Pownall et al, 2002）.

筋分化を決定する細胞内因子として，MyoDファミリー（MyoD, Myf5, myogenin, MRF4）が特に重要である．MyoDは，非筋細胞である繊維芽細胞を筋細胞に分化誘導しうる強力な筋分化決定因子である．MyoDファミリーは総括して筋分化決定因子（myogenic regulatory factor, MRF）と呼ばれ，basic helix-loop-helix構造を持つ転写調節因子である．MyoDとMyf5は，主に初期の体節での細胞系譜の決定に関与し，myogeninは，筋前駆細胞から最終分化し筋管細胞を形成する後期過程に重要だと考えられている（Pownall et al, 2002）.

III. 筋萎縮をきたす神経・筋疾患の治療法開発の現状

1. 筋ジストロフィー

1) 遺伝子治療

筋ジストロフィーの遺伝子治療では，AAVベクターの開発に進展が見られる（Blankinship et al, 2006; Yoshimura et al, 2004）．AAVベクターは，遺伝子導入効率や長期間にわたる導入遺伝子の発現の面で優れている．骨格筋に指向性を持った血清型の同定や，免疫反応を抑えるための工夫がなされている．また，ジストログリカンの糖鎖翻訳後修飾異常には，LARGEと呼ばれる糖転移酵素のアデノウイルスベクターの導入がジストログリカンの機能不全を回復できると報告された（Barresi et al, 2004）.

2) 細胞移植治療

患者本人や家族などのドナーから骨格筋幹細胞を単離し，得られた細胞を骨格筋または静脈注射で筋疾患患者に導入する方法であり，移植細胞が骨格筋に生着し，筋分化することを期待した治療法である．上述のように，筋衛星細胞が生体内での骨格筋分化を担う幹細胞であることが解析さ