Modulation of Thr Phosphorylation of Integrin β₁ during Muscle Differentiation*

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By using transient elevations of cytosolic free calcium levels triggered by integrin antibody or laminin (Kwon, M. S., Park, C. S., Choi, K., Park, C.-S., Ahnn, J., Kim, J. I., Eom, S. H., Kaufman, S. J., and Song, W. K. (2000) Mol. Biol. Cell 11, 1433–1448), we have demonstrated that protein phosphatase 2A (PP2A) is implicated in the regulation of reversible phosphorylation of integrin. In E63 skeletal myoblasts, the treatment of PP2A inhibitors such as okadaic acid and endothall induces an increase of phosphorylation of integrin β₁A and thereby inhibits integrin-induced elevation of cytosolic calcium level and formation of focal adhesions. None of these effects were in differentiated myotubes expressing the alternate β₁D isomorph. In the presence of okadaic acid, PP2A in association with integrin β₁A was reduced on myoblasts, whereas β₁D on myotubes remained bound with PP2A. Both coimmunoprecipitation and in vitro phosphatase assays revealed that dephosphorylation of residues Thr788–Thr789 in the integrin β₁A cytoplasmic domain is dependent upon PP2A activity. Mutational analysis of the cytoplasmic domain and confocal microscopy experiments indicated that substitution of Thr789–Thr789 with Asn789—Asn789 is of critical importance for regulating the function of integrin β₁. These results suggest that PP2A may be a primary regulator of threonine phosphorylation of integrin β₁A and subsequent activation of downstream signaling molecules. Taken together, we propose that dephosphorylation of residues Thr788–Thr789 in the cytoplasmic domain of integrin β₁A may contribute to the linkage of integrins to focal adhesion sites and induce the association with cytoskeleton proteins. The switch of integrin β₁A to β₁D isoform in myotubes therefore may be a mechanism to escape from phospho-regulation by PP2A and promotes a more stable association of the cytoskeleton with the extracellular matrix.

Integrins are heterodimeric transmembrane proteins consisting of α and β subunits and mediate many of the interactions between adjacent cells or between cells and extracellular matrix (ECM).1 The specificity of ligand binding by integrins is conferred by the particular combination of the cytoplasmic domains of α and β subunits (2). The sequences of the cytoplasmic domains of α and β subunits are quite divergent from one another. Alternative forms of cytoplasmic domains that arise from alternate splicing have been found in the integrin β₁, β₃, β₅, β₇, α₅, and α₇ cytoplasmic domains (3–7). Presumably, these diversities within the cytoplasmic domains modulate the capacity of integrins to mediate the transduction of ECM-mediated signals as well as signals arising within the cell that are directed outward via activation of integrins (2, 4, 8–9).

The association of integrins with the cell cytoskeleton and the formation of cell adhesions have been the most widely studied in the aspects of cytoplasmic domain functions. The cytoplasmic domains of the integrin β₁, in particular, have been demonstrated to play central roles in many aspects of integrin function such as cell adhesion, cell migration, control of cell differentiation, proliferation, and programmed cell death. In recent studies (10, 11), more cellular proteins have been reported to directly or indirectly interact with the cytoplasmic domain of integrin β₁. Cytoskeletal proteins such as α-actinin, F-actin, skelemin, talin, vinculin, filamin, and tensin are known to bind the integrin β₁ cytoplasmic domain (12–15). Also, signaling molecules such as focal adhesion kinase, integrin-linked kinase, phosphoinositide 3-kinase, Src, Cas, and novel proteins such as TAP 20 are targeted to adhesion sites where they come into contact with the integrin cytoplasmic domain (16–24).

The spatial relationships and interactions between either cytoskeletal or signaling molecules and the integrin cytoplasmic domain are highly dependent on integrin phosphorylation and can alter the binding affinity of integrins for their respective ligands within the ECM. A variety of studies concerning phosphorylation of integrin cytoplasmic domains has been reported (25–32). Phosphorylated integrin β₁ in src-transformed fibroblasts does not localize to focal contacts (16). Increased phosphorylation of integrin β₁ on Thr residues by addition of calyculin A decreased platelet adhesion and spreading on fibrinogen (34). Mutational analysis of amino acid residues of the β₁ cytoplasmic domain has shown that integrin activity is functionally related to the potential phosphorylation sites of the β₁ cytoplasmic domain. One of the potential sites is composed of two NPYX motifs in the cytoplasmic domains of integrin β₁A, β₁D, β₁E, β₁F, β₁G, and β₁ (35, 36). Upon phosphorylation of the NPYX motifs by tyrosine kinase(s), the integrin loses its affinity for both extracellular ligand and cytoplasmic acid; ETL, endothall; PP2A, protein phosphatase 2A; PP2Ac, protein phosphatase 2A catalytic subunit; [Ca²⁺]ᵢ, cytosolic free calcium concentration; LN, laminin; MHC, myosin heavy chain; RFP, red fluorescence protein; PMSF, phenylmethylsulfonyl fluoride; HS, horse serum; DMEM, Dulbecco’s modified Eagle’s medium; TRITC, tetramethylrhodamine isothiocyanate.

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1 The abbreviations used are: ECM, extracellular matrix; OA, okadaic acid; ETL, endothall; PP2A, protein phosphatase 2A; PP2Ac, protein phosphatase 2A catalytic subunit; [Ca²⁺]ᵢ, cytosolic free calcium concentration; LN, laminin; MHC, myosin heavy chain; RFP, red fluorescence protein; PMSF, phenylmethylsulfonyl fluoride; HS, horse serum; DMEM, Dulbecco’s modified Eagle’s medium; TRITC, tetramethylrhodamine isothiocyanate.
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components of the focal contacts and exits to focal contact. Mutagenesis of Asn or Pro residues of the NPXY motif causes the loss of β subunits from focal contacts. Tyr785 mutation of the NPXY motif of the β1 cytoplasmic domain completely inhibits binding of PAC1 that recognizes integrins in the activated state (37).

In particular, S785D, Y789A, and Y795A resulted in an inability to localize to focal adhesion sites (38). Conversion of the unphosphorylated and phosphorylated state of integrins takes place during cell differentiation. Undifferentiated F9 cells are phosphorylated on the serine residue of the cytoplasmic domain of the β1 chain and dephosphorylated during differentiation (37). During muscle cell differentiation, the integrin β1 undergoes isomeric switch from β1A to β1D (39, 40). Interestingly, the Thr788-Thr789 residues, potential phosphorylation sites in the integrin β1A cytoplasmic domain, are substituted with Asn788/Asn789 in the β1D splice variant.

Although a number of studies have suggested that phosphorylation of β1 integrin cytoplasmic domains alters their functions, there are still numerous conflicting reports regarding the modulation of integrin function. Moreover, relatively little is known about the potential role of Thr phosphorylation (788 – 789) of the integrin β1 cytoplasmic domain and the coordinated activity of protein kinases and phosphatases that regulate threonine phosphorylation in modulating the function of the integrin β1 cytoplasmic domain.

Recently, it was shown that binding of integrin antibodies or laminin (LN) to integrin induces extracellular Ca2+ influx through L-type Ca2+ channels (1) and increases the cytosolic free calcium concentration ([Ca2+]i), which can be used as an indicator of integrin activation (1, 41). In this study, we showed that treatment with PP2A inhibitors such as okadaic acid and endothall inhibits the elevation of [Ca2+]i, and the formation of focal adhesion complex in E63 myoblasts. Also, we demonstrated that PP2A in association with integrin β1 mediates dephosphorylation of residues Thr788-Thr789 on integrin β1A and thereby induces an increase of integrin association with cytoskeleton. In particular, the isomeric switch of β1A to β1D may be a mechanism to escape from phosphorylation by PP2A during skeletal muscle differentiation. The dephosphorylated β1D is likely to provide high tensile strength in association with cytoskeletal proteins for muscle function.

MATERIALS AND METHODS

Antibodies and Reagents—Rabbit anti-integrin β1A and β1D antibody were generated as described previously (42). Anti-PP2A catalytic subunit (PP2Ac) and anti-PP2A B subunit antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-vinculin antibody, anti-myosin heavy chain (MHC) antibody, and ECL reagent were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-vinculin antibody, anti-myosin heavy chain (MHC) antibody, and ECL reagent were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-vinculin antibody was from ImmunoResearch (West Grove, PA). Okadaic acid (OA) and endothall were provided by Craig Kamibayashi (University of Texas). buffalo rat lung (E63) cells were obtained from each pixel in the selected area, whereas average I mean was calculated from I mean. Independent experiments were repeated at least five times with the same gain. Cells exhibiting a calcium influx following treatment with the ionophore A23187 (Sigma) were regarded as viable cells.

Immunoprecipitation and Immunoblotting—E63 cells were washed three times in cold phosphate-buffered saline (PBS) and then lysed with extraction buffer containing 200 mM n-octyl-β-D-glucopyranoside (Sigma), 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM CaCl2, 5 mM MgCl2, 20 μg/ml leupeptin, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 250 μg/ml amphotericin B in a humified atmosphere of 90% air, 5% CO2 at 37 °C as described previously (43). At confluence, differentiation medium (2% HS) was added to induce myotube formation. The expression vectors were transiently introduced into the cells using the LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer’s instructions.

Intracellular Calcium Measurement using Confocal Microscopy—Measurement of [Ca2+]i, by confocal microscopy was performed according to the methods of Kwon et al. (1). Cells cultured on 0.2% (w/v) gelatin-coated coverslips were rinsed twice with bath solution (140 mM NaCl, 5 mM KCl, 25 mM sodium HEPES, pH 7.4) and then incubated in the dark for 1 h at 25 °C in bath solution containing 5 μM fluo-3/AM. Calcium measurements in single cells were made using a Leica (Nussloch, Germany) TCS 4D laser scanning microscope equipped with an argon/krypton laser to excite the dye at 488 nm. Elevation of [Ca2+]i, was initiated by adding the appropriate anti-integrin α1 antibodies (15 μg/ml) to the tissue chamber. Images (512 × 512 pixels) were obtained at a rate of 1 image/s. In order to quantify fluorescence, pixel intensities within the selected single cell area of interest were measured and averaged. The data were analyzed using Microsoft Excel version 4.0 (Redmond, WA). Mean intensity (Imean) was defined as an average of fluorescence intensity obtained from each pixel in the selected area, whereas average I mean was calculated from I mean. Independent experiments were repeated at least five times with the same gain. Cells exhibiting a calcium influx following treatment with the ionophore A23187 (Sigma) were regarded as viable cells.

ppx21 encoding full-length human β1A integrin subunits (56). The integrin β1A was cloned into pDHRed1 (Clontech, Palo Alto, CA) by the unique XhoI and BamHI restriction sites. Mutations were generated by oligonucleotide-paired DNA synthesis using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following primer pairs were generated by PCR: T788N using the forward primer 5′-ATAAGAGTGCCTGAAACACTGGTGCTAATCCG and the reverse primer 5′-CGGTTCCAGAAGAATATATGTTTACGGCTCTT-TAT; T789N using the forward primer 5′-AGAGTGCCTGAAACACTGGTGCTAATCCGGAG and the reverse primer 5′-ATATCTCGAGTGGAC-CACCATGTGTAACGCTCTTCT; T788N/T789N using the forward primer 5′-ATAAGAGTGCCTGAAACACTGGTGCTAATCCGGAG and the reverse primer 5′-CTTCGAGTGGAC-CACCATGTGTAACGCTCTTCT.

To determine the association between PP2A and integrin β1A or β1D in the presence of OA, cells were incubated with 100 μM OA for 2 h at 37 °C. To examine the change in phosphorylation level in the presence of OA, cells were added with 100 μM OA and maintained at 37 °C in serum-free DMEM.

In Vitro Phosphatase Assay—The 4-day-old cultured cells were col-
Fig. 1. Elevation of [Ca\textsuperscript{2+} ], elicited by integrin α, antibody in E63 muscle cells. A, E63 skeletal muscle cells preloaded with fluo-3/AM were treated with integrin α, antibody (O26, 15 μg/ml) or left untreated as a control, and the fluorescence intensity on myoblasts (4-day-old culture) and on myotubes (8-day-old culture) was measured every 3 s using a confocal microscope. Pretreatment of 100 nM OA (n = 20; S.E. = ±0.425–1.336) for 2 h or 100 μM ETL (n = 21; S.E. = ±0.201–0.456) was completely blocked the integrin α,β,–mediated [Ca\textsuperscript{2+} ] elevation in myoblasts (red dotted line) and did not block the elevation of [Ca\textsuperscript{2+} ], in fully differentiated myotubes (red solid line). Exposure to buffer had no effect on [Ca\textsuperscript{2+} ]. The values depicted are the averages of fluorescence intensity obtained from at least 20 single cells in at least five independent experiments conducted under identical experimental conditions. B, a and d show the elevated [Ca\textsuperscript{2+} ] within 40 s following exposure to integrin α, antibody (15 μg/ml), the b and c were obtained in the presence of 100 nM OA, and c and f were obtained in the presence of 100 μM ETL. Cells shown in a–c were grown for 4 days (myoblasts, MB) and in the d–f were grown for 8 days (myotubes, MT). Bar, 50 μm.

RESULTS

PP2A Inhibitors Block the Elevation of [Ca\textsuperscript{2+} ], in E63 Myoblasts—Integrin ligation has been shown to result in activation of a variety of cellular signaling pathways (8, 44, 45). In skeletal muscle cells, we demonstrated previously (1) that binding of integrin α, antibody or LN to integrin induced both Ca\textsuperscript{2+} release from inositol 1,4,5-trisphosphate-sensitive SR Ca\textsuperscript{2+} stores and extracellular Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels. Changes in the cytosolic free calcium concentration ([Ca\textsuperscript{2+} ]) mediated by integrin have been reported in a variety of cell types and can be used as an indicator of integrin activation (1, 41). By utilizing integrin antibody/LN-induced changes of [Ca\textsuperscript{2+} ], we investigated the relationship between integrin activation and the relative state of phosphorylation of the in-
Integrin cytoplasmic domain. E63 myogenic cells elicited the elevation of $[\text{Ca}^{2+}]_{i}$ by the addition of integrin $\beta_{1\text{A}}$ antibody (O26, 15 $\mu$g/ml) (1), which specifically binds to the extracellular domain of the integrin $\beta_{1\text{A}}$ subunit, or by the addition of LN (100 $\mu$g/ml). The elevation of $[\text{Ca}^{2+}]_{i}$ in undifferentiated myoblasts (4-day-old culture, Fig. 1, A and B) was completely blocked by pretreatment with OA (100 nM) or ETL (100 $\mu$M), Ser/Thr-phosphatase inhibitors (46, 47). In contrast, the pretreatment with OA or ETL on differentiated myotubes (8-day culture) did not inhibit the integrin-mediated $[\text{Ca}^{2+}]_{i}$ elevation (Fig. 1, A and B). The inhibitory effect of OA or ETL evident in these experiment, therefore, indicates that integrin activation is closely related to its phosphorylation status (48), and the differential sensitivity of myoblasts and myotubes to these phosphatase inhibitors may be due to the different regulatory mechanisms controlling phosphorylation and/or due to the presence of different integrin isoforms.

**Fig. 2. Effect of okadaic acid on integrin $\beta_{1}$ phosphorylation.** A, during muscle differentiation, the expression of integrin $\beta_{1\text{A}}$ and $\beta_{1\text{D}}$ was examined by immunoblotting (IB). As phenotypic markers indicating myogenic differentiation, muscle-specific protein, myosin heavy chain was detected. B, sequence comparison of integrin $\beta_{1\text{A}}$ and $\beta_{1\text{D}}$ cytoplasmic domains. Potential Ser/Thr phosphorylation sites are indicated by asterisks. C, the 4-day-old cultured cells were incubated with or without 100 nM OA for 4 h, followed by immunoprecipitation with anti-integrin $\beta_{1\text{A}}$ and $\beta_{1\text{D}}$ antibodies, followed by immunoblot analysis with anti-phosphothreonine (P-thr) antibody. To verify equal loading amounts of protein, the blot was reprobed with anti-integrin $\beta_{1\text{A}}$ or $\beta_{1\text{D}}$ antibodies. D, threonine phosphorylation of integrin $\beta_{1\text{A}}$ was analyzed with the antibody that specifically recognizes the phosphorylated threonine at 788–789 residues in the integrin $\beta_{1\text{A}}$. The 4-day-old cultured cell treated with 100 nM OA and 100 $\mu$M ETL were immunoprecipitated with anti-integrin $\beta_{1\text{A}}$ antibody and followed by immunoblotting with anti-phosphothreonine (P-thr) antibody. To verify equal loading amounts of protein, the immunoprecipitates were blotted with anti-integrin $\beta_{1\text{A}}$ antibody.

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Due, one of potential phosphorylation sites in the integrin β1A cytoplasmic domain, are substituted with Asn788–Asn789 in the β1D splice variant (Fig. 2B). To assess the effects of OA upon Thr phosphorylation of integrin β1, we preincubated 4-day-old cultured myoblasts with 100 nM OA for 4 h. This treatment had no effect upon either cell viability or the synthesis of the integrin β1 (not shown). As shown in Fig. 2C, integrin β1A seem to be Thr-phosphorylated at a relatively low level in 4-day-old cultured cells. However, OA treatment resulted in a large increase of Thr-phosphorylated integrin β1A. Interestingly, in the presence of OA, the level of Thr phosphorylation of integrin β1D, another splice variant of integrin β1, was unchanged. To identify threonine phosphorylation on integrin β1A, we performed immunoblot analysis with the antibody that specifically recognizes the phosphorylated threonine at 788–789 residues in the cytoplasmic domain of integrin β1A. The level of Thr788–Thr789 phosphorylation of integrin β1A was increased in the presence of OA or ETL (Fig. 2D), suggesting that threonine 788–789 residues could be primarily phosphorylation sites in integrin β1A. The insensitivity of myotubes to OA is likely due to the unphosphorylated state of β1D since the Thr788–Thr789 residues are substituted with Asn788–Asn789 in the β1D splicing variant. Therefore, the alternative splicing that generates alternative integrin β1 cytoplasmic domains may be an important mechanism to facilitate regulation of integrin activity that depends upon its unphosphorylated or phosphorylated state.

Differential Association of PP2A with Integrin β1A and β1D during Myogenic Differentiation—In order to identify endogenous candidate phosphatases responsible for dephosphorylation of integrin β1A, cell lysates of 4-day-old cultured cells were immunoprecipitated with specific antibodies to the integrin β1A and β1D and then immunoblotted with PP2A catalytic subunit (PP2Ac) antibody. As shown in Fig. 3, integrin β1A and β1D appeared to be associated with PP2Ac. The expression of PP2A was unchanged during E63 muscle cell differentiation (data not shown). The association of PP2Ac with integrin β1A was dramatically decreased in OA-treated myoblasts (Fig. 3A). The association of the integrin β1A with PP2Ac therefore seems to be highly dependent upon the phosphorylated states of the integrin. However, OA did not affect the association of β1D with PP2Ac on either myoblasts or myotubes. These data suggest that dephosphorylation of integrin β1A is regulated, at least in part, by the action of an OA-sensitive phosphatase PP2Ac.

To identify PP2A regulatory subunits in association with integrin β1A or β1D, E63 cell lysates were immunoprecipitated with PP2A regulatory subunits, B, B’, and B” antibodies, and immunoblotted by integrin β1A or β1D antibodies. B, B’, and B” regulatory subunits showed differential affinities for the integrin β1A and β1D. B regulatory subunit was predominantly associated with β1A but not β1D, and B” subunit was weakly interacted with both integrin β1A and β1D. B” subunit appeared to be associated with both integrin β1A and β1D. In addition, the association of B subunit to integrin β1A was dramatically decreased in the presence of OA, and association of B” subunit to integrin β1A or β1D remains bound even in the presence of OA. The dissociation of B subunit with integrin β1A by pretreatment of OA is similar to that of PP2Ac with integrin β1A. It suggests that differential association of B subunit of PP2A with integrin β1A and β1D may be related to the myogenic differentiation, and B subunit of PP2A may participate in the dephosphorylation of integrin β1A.

PP2A Dephosphorylates Residues Thr788–Thr789 in the Cytoplasmic Domain of Integrin β1A—To further confirm that PP2A mediates dephosphorylation of the integrin β1 cytoplasmic domain, in vitro phosphatase activity assays were conducted with synthetic phosphopeptides corresponding to the potential O-linked phosphorylation sites in the cytoplasmic domain of integrin β1A and β1D, i.e., Thr777, Ser785, Thr788, and Thr789 (Fig. 4A). Cell lysates of 4-day-old cultured cells were used as a source of crude PP2A. Incubation of β1A1, β1A2, and β1D1 phosphopeptides with crude PP2A or purified PP2A AC dimer resulted in the release of phosphate, and addition of OA completely blocked its release in a dose-dependent manner (Fig. 4, B and C). In contrast, crude PP2A and PP2A AC dimer did not exhibit any phosphatase activity toward β1A1, β1D1, or β1D2 phosphopeptides and exhibited only a low phosphatase activity against β1A2 phosphopeptide. In order to inhibit the tyrosine...
phosphatase activity of PP2A, 1 mM vanadate was included in the incubation with the phosphopeptides. In this reaction, phosphate release was comparable to control (Fig. 4, B and C), indicating that the release of phosphate is due to specific Thr phosphatase activity. To identify PP2A regulatory subunits mediating the dephosphorylation of integrin $\beta_1$A, cells were immunoprecipitated with anti-B regulatory subunit antibody, and the immunocomplexes were used as an enzyme source for phosphatase activity assay. To verify the binding of PP2A with integrin $\beta_1$, the immunocomplexes were probed with anti-PP2Ac antibody. The released phosphates were detected by color reaction with molybdate dye. The control reaction (Rxn) indicates the absence of inhibitors.

![Diagram](https://example.com/diagram.png)

**Fig. 4.** PP2A mediates dephosphorylation of residues Thr$^{788}$-Thr$^{789}$ in integrin $\beta_1$A. A, schematic representation of integrin $\beta_1$A and $\beta_1$A1 phosphateptides. B and C, the phosphopeptides were reacted with the crude PP2A (B) or purified PP2A AC dimer (C) in the presence of inhibitors including vanadate (Va) or okadaic acid (OA) as described under "Materials and Methods." D, cells (4-day-old) were immunoprecipitated with anti-B regulatory subunit antibody or without antibody (Con), and the immunocomplexes were used as an enzyme ($E_{N2}$) source for phosphatase activity assay. To verify the binding of PP2A with integrin $\beta_1$, the immunocomplexes were probed with anti-PP2Ac antibody. The released phosphates were detected by color reaction with molybdate dye. The control reaction (Rxn) indicates the absence of inhibitors.

Okadaic Acid Does Not Block Integrin-mediated [Ca$^{2+}$], Elevation in the $\beta_1$A Mutant-transfected Myoblasts—To test the function of two consecutive threonines in the cytoplasmic domain of integrin $\beta_1$A, the B subunit of PP2A seems to participate in this process.

Results indicate that PP2A may interact directly with these phosphopeptides and mediate, at least in part, the release of phosphate from residues Thr$^{788}$-Thr$^{789}$ in the cytoplasmic domain of integrin $\beta_1$A, and the B subunit of PP2A seems to participate in this process.
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Fig. 5. Comparison of the ability of integrin $\beta_{1A}$ mutants and $\beta_{1A}$ wild type to elevate cytosolic free calcium in E63 myoblasts. A series of $\beta_{1A}$ mutants was constructed in pDsRed1-N1 (RFP) vector. The E63 myoblasts were transiently transfected with RFP vector, RFP vector containing $\beta_{1A}$ wild-type or $\beta_{1A}$ mutants as indicated. E63 myoblasts preloaded with fluo-3/AM were treated with integrin agonist OA. However, the elevation of $[Ca^{2+}]_{i}$ was triggered by integrin stimulation. Calcium influx by treatment of integrin agonist OA or ETL treatment of myoblasts (4-day-old culture; Fig. 6, a–c). The cultured E63 cells were treated for 4 h with 100 nM OA or with 100 $\mu$M ETL for 2 h. The formation of focal adhesions was dramatically decreased by OA or ETL treatment of myoblasts (4-day-old culture; Fig. 6, a–c). The values depicted are the averages of fluorescence intensity obtained from at least 20 single cells in at least five independent experiments conducted under identical experimental conditions.

Fig. 6. Effect of okadaic acid or endothall on the focal adhesion formations. The cultured E63 cells were treated for 4 h with 100 nM OA or with 100 $\mu$M ETL for 2 h. The formation of focal adhesions was visualized by staining with anti-vinculin (Vin) or anti-paxillin (Pax) antibodies. Formation of focal adhesions was dramatically decreased by OA or ETL treatment of myoblasts (4-day-old culture; b, c, e, and f), and no effects were evident on myotubes (8-day-old culture; h, i, k, and l). Bar, 30 $\mu$m.

calcium influx by treatment of integrin $\alpha_7$ antibody (O26) using confocal microscopic analysis. In E63 myoblasts transfected with RFP vector or RFP-$\beta_1$ wild-type, the elevation of $[Ca^{2+}]_{i}$ was triggered by integrin $\alpha_7$ antibody, and the elevation of $[Ca^{2+}]_{i}$ was completely inhibited by pretreatment with 100 nM OA. However, the elevation of $[Ca^{2+}]_{i}$ was not affected even in the presence of OA in the three mutants, T788N, T789N, and T788N/T789N (Fig. 5). In addition, the confocal microscopic analysis confirmed that the level of elevated $[Ca^{2+}]_{i}$ in T788N- or T789N-transfected myoblasts was not much different from that in T788N/T789N-transfected myoblasts. Therefore, these results indicate that substitution of Thr$^{788}$–Thr$^{789}$ with Asn$^{788}$–Asn$^{789}$ in the $\beta_{1A}$ is at least able to mimic the $\beta_{1D}$ isoform.

PP2A Inhibitors Inhibit the Formation of Focal Adhesions in E63 Myoblasts—It has been reported that the binding of integrin either with antibodies or its ligands promotes its association with the cell cytoskeleton (7), and the formation of focal adhesion is highly dependent upon phosphorylation of integrin $\beta_1$ (11, 16, 21, 23–32, 37–40). To address the formation of focal adhesions depending upon phosphorylation of integrin $\beta_1$ cells were treated with PP2A inhibitor such as OA or ETL, and the formation of focal adhesions was visualized by staining with anti-vinculin or anti-paxillin antibodies. Myoblasts treated with OA or ETL exhibited the dramatic reduction of focal adhesions containing vinculin and paxillin (Fig. 6, b, c, e, and f). However, myotubes that predominantly express integrin $\beta_{1D}$ isoform were unchanged in the formation of focal adhesions even in the presence of OA or ETL (Fig. 6, h, i, k, and l). These data reflect that phosphorylation of residues Thr$^{788}$–Thr$^{789}$ in the $\beta_{1A}$ cytoplasmic domain results in integrin inactivation and promotes dissociation from cytoskeleton proteins. The alternatively spliced integrin $\beta_{1D}$ (T788N/T789N) may therefore represent a mechanism to escape from the regulation of PP2A activity and thereby display an increased affinity for both ECM and cytoskeleton. These results are consistent with observations in integrin $\beta_{1D}$-transfected cells, which exhibit targeting of the transfected integrin $\beta_{1D}$ into focal adhesions with subsequent displacement of the endogenous $\beta_{1A}$ and an elevated stability of focal adhesions (40, 56).

DISCUSSION

In this study, we have analyzed the regulation of integrin $\beta_1$ phosphorylation in skeletal muscle cells. Our studies demonstrate that pretreatment of protein serine/threonine phosphatase inhibitors such as OA or ETL inhibits the elevation of $[Ca^{2+}]_{i}$, and formation of focal adhesions. Integrin $\beta_{1A}$ is associated with the PP2A, and its association with PP2A seems to be dependent upon the phosphorylation status of the $\beta_{1A}$ cyto-

Ctrl OA ETL

Pax

Vin

Pax

Vin

MB

MT

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plasmic domain. Furthermore, an in vitro phosphatase assay indicates that two consecutive residues Thr785–Thr789 in the cytoplasmic domain of integrin β1A are specifically dephosphorylated by PP2A.

Although OA treatment results in increased phosphorylation of integrin β1A, it may also result in Ser/Thr phosphorylation of a variety of proteins as a result of diverse PP2A activity (47, 48, 57, 58). However, the increase of integrin β1A phosphorylation does indicate that integrin β1A has PP2A-sensitive dephosphorylation sites. In addition, in vitro phosphatase assay and immunoblotting data clearly proved that the phosphate of the Thr785–Thr789 residues on integrin β1A is specifically released by the action of PP2A and that dephosphorylation of Thr785–Thr789 residues results in enhanced focal adhesion formation.

However, there are conflicting reports that Thr785–Thr789 mutant was defective in mediating cell attachment and did not contribute to fibronectin formation (29), and mutation of the three consecutive threonines in integrin β3 to alanines strongly inhibits the integrin functions (59). Even though several β subunits have been reported to become phosphorylated or unphosphorylated, the cellular functions of the phosphorylation are still controversial. The conservation of the region corresponding to Thr785–Thr789 residues between the cytoplasmic domains of the integrin β subunits, however, indicates that it is important for a general cellular function of the integrin β subunits.

Reversible phosphorylation of integrin β1 is known to be involved in a variety of cellular functions such as formation of focal adhesions, association with cytoskeletal components, and signal transduction (11, 16, 21, 35, 37–38). Mutation studies of integrin β1 cytoplasmic domains have focused mainly on the possible role of dephosphorylation sites along integrin β1A. It was shown that PP2A is associated with the integrin β1A. PP2A can accumulate at focal contact sites due to its interaction with paxillin and a truncated isoform of the PP2A B56 subunit promotes cell motility through paxillin phosphorylation (60). The activation of PP2A may participate in many physiological consequences in signal transduction during myocyte differentiation since β1A, but not β1D, interacts with the Nck signaling protein (42).

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