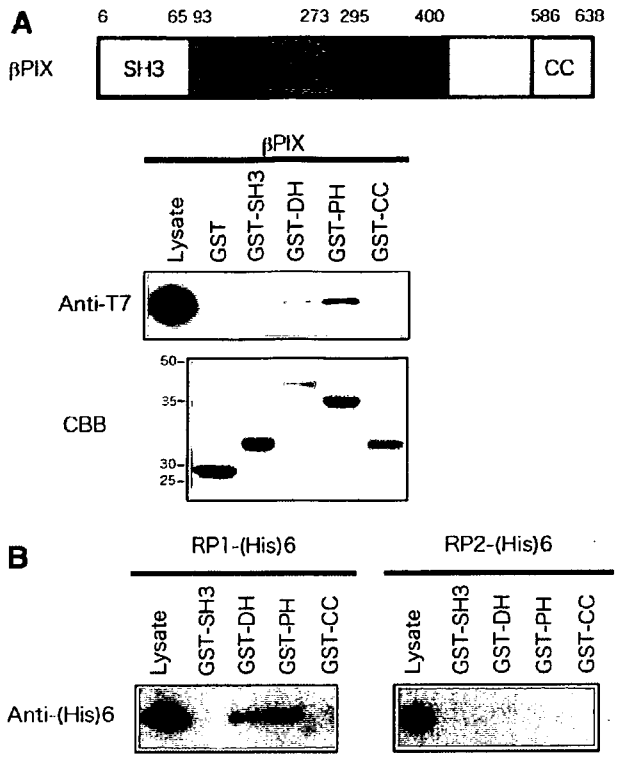

Fig. 3. Identification of affixin-binding domain in βPIX by pulldown assay. (A) Cell lysates from COS-7 cells overexpressing T7-tagged affixin were incubated with glutathione Sepharose 4B beads bound to GST, GST-SH3, GST-DH, GST-PH and GST-CC. Domain structures of βPIX are shown at the top. After overnight incubation at 4 °C, the beads were washed and bound proteins were subjected to immunoblotting with anti-T7 polyclonal antibody. A band at 50 kDa, which corresponds to T7-affixin, was detected in GST-DH and GST-PH. GST fusion proteins used for the pulldown assay were stained with coomasie brilliant blue. GST, GST-SH3, GST-DH, GST-PH and GST-CC domains have molecular masses of 26, 32.8, 46.6, 38 and 32.3 kDa, respectively. (B) Bacterially expressed $(His)_6$-tagged RP1 and RP2 were purified and incubated with GST-βPIX domains as described above. The proteins bound to GST fusion proteins were subjected to immunoblotting using anti-$(His)_6$ antibody.

ing T7-affixin were incubated with GST fusion proteins carrying each domain of human βPIX bound to glutathione Sepharose 4B. Fig. 3A shows that the DH and PH domains of βPIX can interact with T7-affixin, whereas the SH3 and CC domains of βPIX failed. PH domain of βPIX showed higher affinity to T7-affixin than DH domain of βPIX. To examine whether affixin-RP1 directly binds to DH and PH domains of βPIX, pulldown assay was performed using bacterially expressed $(His)_6$-tagged RP1 instead of cell lysate. As shown in Fig. 3B, $(His)_6$-tagged RP1 was bound to DH and PH domains of βPIX, whereas $(His)_6$-tagged RP2 failed to interact with any domain of βPIX. PH domain of βPIX showed higher affinity to $(His)_6$-tagged RP1 than DH domain of βPIX. These results were consistent with the result of a pulldown assay using cell lysates and suggest direct interaction between affixin and βPIX.

3.4. Rac1 but not Cdc42 is activated in C2C12-affixin cells

The reorganization of cytoskeletal actin is regulated by Rho family small GTPases. βPIX is known as a specific GEF for

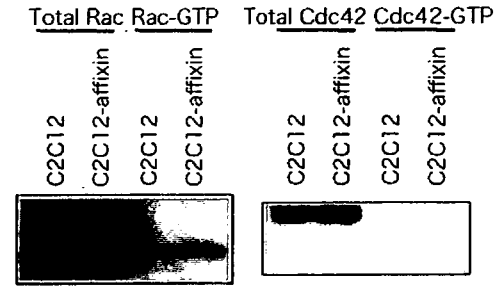

Fig. 4. Pulldown assay of active Rac1 and Cdc42. C2C12-affixin and C2C12 cells were lysed and incubated with glutathione Sepharose 4B conjugated with GST-p21-binding (CRIB) domain (residues 67–150) of human PAK-1. The CRIB domain specifically associates with Rac-GTP and Cdc42-GTP. The total amounts of Rac1 and Cdc42 were assessed by the immunoblotting using the cell lysates.

Rac1/Cdc42 and induces membrane ruffling [13]. To examine whether Rac1/Cdc42 is activated in the C2C12-affixin cells, a pulldown assay was performed using GST-fusion protein derived from PAK, the effector protein of Rac1 and Cdc42. Cell lysates from C2C12-affixin and C2C12 cells were incubated with GST-p21 binding domain of PAK bound to glutathione Sepharose 4B. This analysis revealed increased level of Rac1-GTP in the C2C12-affixin cells (Fig. 4). There was no significant difference in Cdc42-GTP. The total expression levels of Rac1 and Cdc42 protein in the C2C12-affixin were equivalent to the C2C12 cells. These results indicate that the exogenous expression of affixin can activate Rac1 but not Cdc42 in C2C12 cells.

3.5. Dominant-negative βPIX suppresses lamellipodium formation in the C2C12-affixin cells

Activation of Rac1 is mediated by GEFs including βPIX [20]. To examine the involvement of βPIX in lamellipodium formation observed in the C2C12-affixin cells, the HA-tagged dominant negative mutant of βPIX were introduced into the C2C12-affixin cells. Exogenous βPIX and affixin were labelled with anti-HA and anti-T7 antibodies, respectively. As shown in Fig. 5, the C2C12-affixin cells overexpressing wild type βPIX form lamellipodia as observed in the C2C12-affixin cells without transfection. In contrast, dominant negative mutant of βPIX (L238R, L239S) suppressed the lamellipodium formation in the C2C12-affixin cells. The association of dominant negative βPIX and affixin was confirmed by an immunoprecipitation study using COS-7 cells (data not shown). Similar inhibition of lamellipodium formation was observed in the C2C12-affixin cells transfected with dominant negative mutant of αPIX. In C2C12-affixin cells transiently transfected with dominant negative mutants of PIX, expression level of affixin, α and βPIXs was equivalent to C2C12-affixin cells without transfection on immunoblot (data not shown). These results suggest that both α and βPIXs are necessary for the lamellipodium formation in the C2C12-affixin cells.

4. Discussion

Lamellipodium is the dynamic actin-based structure and its formation is mediated by the activation of Rho family small GTPases and their effector proteins. Small Rho GTPases are activated by GEFs, which catalyze exchange of GDP for

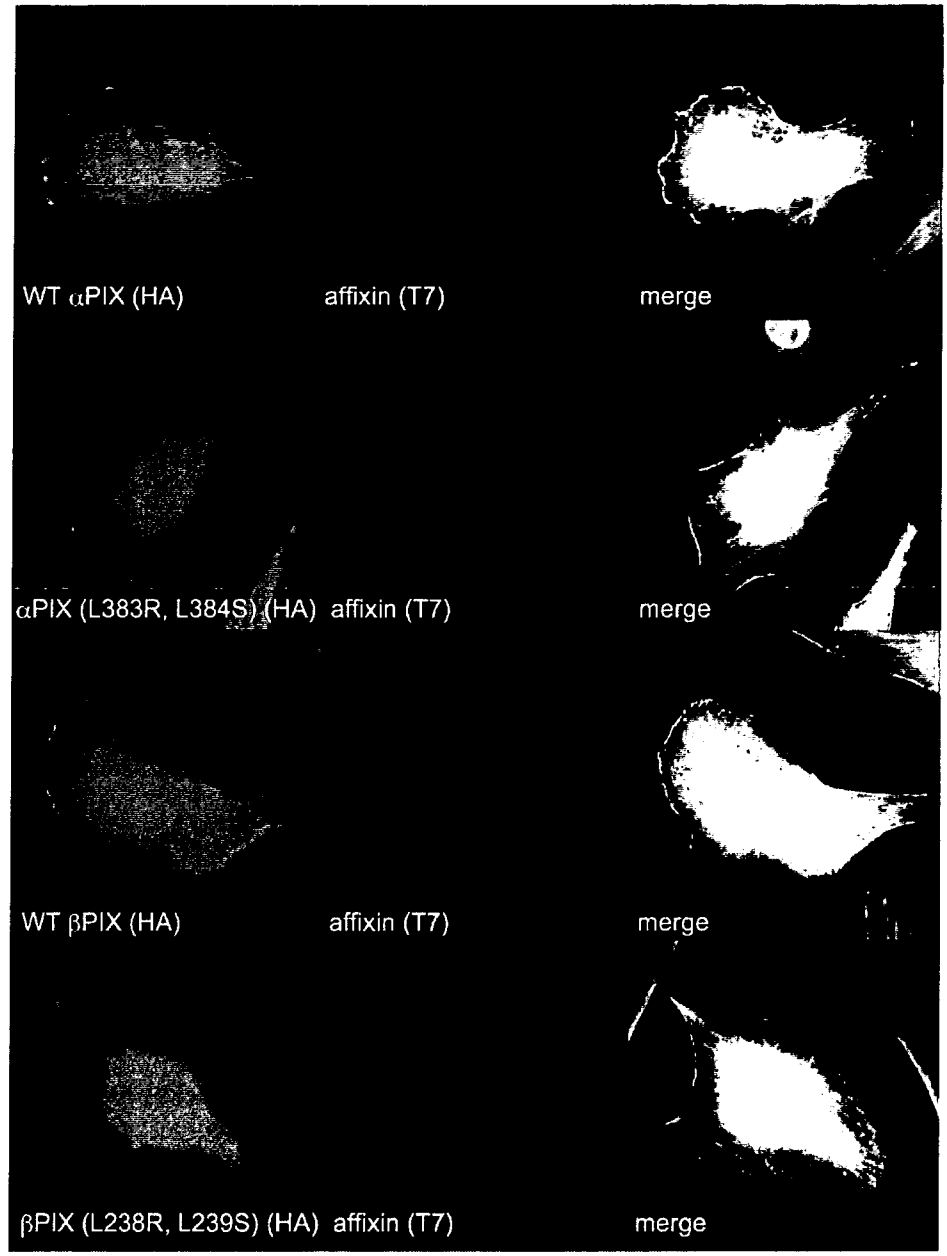

Fig. 5. Immunofluorescence analysis of the C2C12-affixin cells transformed with the dominant negative mutants of PIXs. The C2C12-affixin cells were transfected with wild type or dominant negative mutants of PIXs, αPIX (L383R, L384S) and βPIX (L238R, L239S). Exogenous PIX and affixin were simultaneously immunolabeled with anti-HA and anti-T7 antibodies, respectively. Scale bar, 20 μm.

GTP. GEFs are regulated by protein–protein interaction, oligomerization and relief of intramolecular inhibitory sequence [21]. More than 60 GEFs including PIXs have been identified in human genomic sequences [22]. βPIX expression levels are highest in skeletal muscle by Northern blot analysis using KIAA clones as probes (KIAA0142, http://www.kazusa.or.jp/huge/gfimage/northern/html/KIAA0142.html).

In C2C12 cells, exogenous expression of affixin induced prominent lamellipodia. Accumulated βPIX, together with ILK was observed at the tips of lammelipodia and co-localized with affixin. The interaction of affixin and βPIX was confirmed by immmunoprecipitation and pulldown assays. These data suggest that affixin could promote reorganization of subsarcolemmal actin cytoskeleton associated with accumulation of GEFs.

We previously demonstrated that Madin-Darby canine kidney cells overexpressing CH1 domain of affixin formed membrane protrusions, while control cells transfected with lacZ showed a cobble-like morphology [6]. This CH1-induced reorganization of cytoskeletal actin is mediated by the activation of both Rac1 and Cdc42 through αPIX. Interestingly, only Rac1 but not Cdc42 is activated in the C2C12-affixin cells used for this study. αPIX has been reported to contain Rac-specific interaction domain at C-terminus of PH domain [23]. When

αPIXs form a homodimer, the DH domain of one molecule and Rac-specific interaction domain of another molecule work together and bind Rac specifically, whereas monomeric αPIX can interact with Cdc42 as well as Rac. Despite the lack of a Rac-specific interaction domain, βPIX binds directly and activates Rac1 specifically but not Cdc42 in human embryonal kidney 293 cells [24]. Previous study using yeast two hybrid system [18,19] and our immunoprecipitation results have shown that α and βPIX form heterodimers in the C2C12-affixin cells (Fig. 2A), although their specificity for small GTPase remains unclear. We have also shown that lamellipodium formation in the C2C12-affixin cells was inhibited by overexpression of dominant negative forms of α or βPIX (Fig. 5). From these results, we suspect that the DH domain of βPIX and the Rac-specific interacting domain of αPIX work together and activate specifically Rac1. Further analyses are needed to elucidate the precise regulation of GEF activity of both PIXs in C2C12-affixin cells.

Lamellipodium formation is essential for cell motility. In epithelial cell monolayer, the cells around the wounded edge form lamellipodia and activation of Rac1 but not Cdc42 or Rho is required for wound closure [25]. Similarly, activated satellite cells form lamellipodia and migrate around the injured lesion during skeletal muscle regeneration [26]. Very recently, dysferlin reportedly localized in the T-tubule system of differentiating C2C12 cells and was recruited to the wounded site [27]. Dysferlin is known to have an important role in skeletal muscle wound healing [11]. In response to the sarcolemmal injury of muscle fibers, dysferlin patch was formed around wounded sites for resealing in a calcium-dependent manner. Calcium-dependent membrane resealing is also reported in wounded *Xenopus* oocyte, where Cdc42 and RhoA are activated [28]. Membrane repair is composed of two process: plasma membrane resealing and reorganization of subsarcolemmal cytoskeleton [29]. Accumulation of dysferlin and activation of Rac1 via PIXs in the lamellipodia of C2C12-affixin cells may suggest the involvement of these molecules in sarcolemmal repair followed by cytoskeletal actin reorganization.

In conclusion, exogenous expression of affixin promotes lamellipodium formation in C2C12 myoblasts via activation of Rac1 by α and βPIXs. Reorganization of cytoskeletal actin mediated by affixin may be involved in the skeletal muscle dysferlin-related membrane repair system.

Acknowledgements: This work was supported by Grants-in-aid for Scientific Research (C.M.; C, 15590924, 18590966) (Y.K.H.; B, 18390265), and Grant-in-Aid for Exploratory Research (Y.K.H.; 50238135) from the Japan Society for the Promotion of Science, the Nakatomi Foundation (C.M.), the Research on Psychiatric and Neurological Diseases and Mental Health from Health and Labor Sciences Research Grants (Y.K.H. and I.N.), and the Research Grant for Nervous and Mental Disorders, from the Ministry of Health, Labour and Welfare (I.N.).

References

[1] Olski, T.M., Noegel, A.A. and Korenbaum, E. (2001) Parvin, a 42 kDa focal adhesion protein, related to the alpha-actinin superfamily. J. Cell Sci. 114, 525–538.
[2] Yamaji, S. et al. (2001) A novel integrin-linked kinase-binding protein, affixin, is involved in the early stage of cell-substrate interaction. J. Cell Biol. 153, 1251–1264.
[3] Nikolopoulos, S.N. and Turner, C.E. (2000) Actopaxin, a new focal adhesion protein that binds paxillin LD motifs and actin and regulates cell adhesion. J. Cell Biol. 151, 1435–1448.
[4] Tu, Y., Huang, Y., Zhang, Y., Hua, Y. and Wu, C. (2001) A new focal adhesion protein that interacts with integrin-linked kinase and regulates cell adhesion and spreading. J. Cell Biol. 153, 585–598.
[5] Sepulveda, J.L. and Wu, C. (2006) The parvins. Cell. Mol. Life Sci. 63, 25–35.
[6] Mishima, W. et al. (2004) The first CH domain of affixin activates Cdc42 and Rac1 through alphaPIX, a Cdc42/Rac1-specific guanine nucleotide exchanging factor. Gene Cell 9, 193–204.
[7] Yamaji, S. et al. (2004) Affixin interacts with alpha-actinin and mediates integrin signaling for reorganization of F-actin induced by initial cell–substrate interaction. J. Cell Biol. 165, 539–551.
[8] Matsuda, C. et al. (2005) Dysferlin interacts with affixin (beta-parvin) at the sarcolemma. J. Neuropathol. Exp. Neurol. 64, 334–340.
[9] Liu, J. et al. (1998) Dysferlin, a novel skeletal muscle gene, is mutated in Miyoshi myopathy and limb girdle muscular dystrophy. Nat. Genet. 20, 31–36.
[10] Bashir, R. et al. (1998) A gene related to *Caenorhabditis elegans* spermatogenesis factor fer-1 is mutated in limb-girdle muscular dystrophy type 2B. Nat. Genet. 20, 37–42.
[11] Bansal, D., Miyake, K., Vogel, S.S., Groh, S., Chen, C.C., Williamson, R., McNeil, P.L. and Campbell, K.P. (2003) Defective membrane repair in dysferlin-deficient muscular dystrophy. Nature 423, 168–172.
[12] Yaffe, D. and Saxel, O. (1977) Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature 270, 725–727.
[13] Manser, E. et al. (1998) PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. Mol. Cell 1, 183–192.
[14] Matsuda, C., Aoki, M., Hayashi, Y.K., Ho, M.F., Arahata, K. and Brown Jr., R.H. (1999) Dysferlin is a surface membrane-associated protein that is absent in Miyoshi myopathy. Neurology 53, 1119–1122.
[15] Sotgia, F. et al. (2000) Caveolin-3 directly interacts with the C-terminal tail of beta -dystroglycan. Identification of a central WW-like domain within caveolin family members. J. Biol. Chem. 275, 38048–38058.
[16] del Pozo, M.A., Price, L.S., Alderson, N.B., Ren, X.D. and Schwartz, M.A. (2000) Adhesion to the extracellular matrix regulates the coupling of the small GTPase Rac to its effector PAK. EMBO J. 19, 2008–2014.
[17] Hart, M.J., Eva, A., Zangrilli, D., Aaronson, S.A., Evans, T., Cerione, R.A. and Zheng, Y. (1994) Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product. J. Biol. Chem. 269, 62–65.
[18] Rosenberger, G., Jantke, I., Gal, A. and Kutsche, K. (2003) Interaction of alphaPIX (ARHGEF6) with beta-parvin (PARVB) suggests an involvement of alphaPIX in integrin-mediated signaling. Hum. Mol. Genet. 12, 155–167.
[19] Koh, C.G., Manser, E., Zhao, Z.S., Ng, C.P. and Lim, L. (2001) Beta1PIX, the PAK-interacting exchange factor, requires localization via a coiled-coil region to promote microvillus-like structures and membrane ruffles. J. Cell Sci. 114, 4239–4251.
[20] Scita, G., Tenca, P., Frittoli, E., Tocchetti, A., Innocenti, M., Giardina, G. and Di Fiore, P.P. (2000) Signaling from Ras to Rac and beyond: not just a matter of GEFs. EMBO J. 19, 2393–2398.
[21] Schmidt, A. and Hall, A. (2002) Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Gene Dev. 16, 1587–1609.
[22] Venter, J.C. et al. (2001) The sequence of the human genome. Science 291, 1304–1351.
[23] Feng, Q., Baird, D. and Cerione, R.A. (2004) Novel regulatory mechanisms for the Dbl family guanine nucleotide exchange factor Cool-2/alpha-Pix. EMBO J. 23, 3492–3504.
[24] ten Klooster, J.P., Jaffer, Z.M., Chernoff, J. and Hordijk, P.L. (2006) Targeting and activation of Rac1 are mediated by the exchange factor beta-Pix. J. Cell Biol. 172, 759–769.

[25] Fenteany, G., Janmey, P.A. and Stossel, T.P. (2000) Signaling pathways and cell mechanics involved in wound closure by epithelial cell sheets. Curr. Biol. 10, 831–838.
[26] Bischoff, R. and Flanzini-Armstrong, C. (2004) Stellite and stem cells in muscle regeneration. Myology, 66–86.
[27] Klinge, L., Laval, S., Keers, S., Haldane, F., Straub, V., Barresi, R. and Bushby, K. (2007) From T-tubule to sarcolemma: damage-induced dysferlin translocation in early myogenesis. FASEB J.
[28] Benink, H.A. and Bement, W.M. (2005) Concentric zones of active RhoA and Cdc42 around single cell wounds. J. Cell Biol. 168, 429–439.
[29] Bement, W.M., Yu, H.Y., Burkel, B.M., Vaughan, E.M. and Clark, A.G. (2007) Rehabilitation and the single cell. Curr. Opin. Cell Biol. 19, 95–100.