Involvement of the nectin-afadin complex in PDGF-induced cell survival

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Summary

The nectin-afadin complex is involved in the formation of cell-cell junctions, such as adherens junctions (AJs) and tight junctions (TJs). Nectins are Ca^{2+}-independent immunoglobulin-like cell-cell adhesion molecules, whereas afadin is an intracellular nectin-binding protein that connects nectins to the cadherin-catenin system at AJs and to the claudin–zona-occludens (ZO) protein system at TJs. Afadin$^{-/-}$ mice show embryonic lethality, resulting from impaired migration and improper differentiation of cells due to disorganization of cell-cell junctions during gastrulation. However, it remains to be elucidated whether disruption of afadin affects apoptosis. In the present study, we first found that embryoid bodies derived from afadin-knockout embryonic stem (ES) cells contained many more apoptotic cells than those derived from wild-type ES cells. We also revealed that apoptosis induced by serum starvation or Fas-ligand stimulation was increased in cultured NIH3T3 cells when afadin or nectin-3 was knocked down. The nectin-afadin complex was involved in the platelet-derived growth factor (PDGF)-induced activation of phosphatidylinositol 3-kinase (PI3K)-Akt signaling for cell survival. This complex was associated with PDGF receptor on the plasma membrane at cell-cell adhesion sites. Thus, the nectin-afadin complex is involved in PDGF-induced cell survival, at least through the PI3K-Akt signaling pathway.

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Key words: Afadin, Akt, Cell survival, Growth factor, Nectin, Phosphatidylinositol 3-kinase

Introduction

Cell-cell adhesion is a fundamental feature for multicellular organisms because all of their physiological functions are crucially dependent on it: its loss results in death. In polarized epithelial cells, cell-cell adhesion is mediated by specialized junctional complexes comprised of tight junctions (TJs), adherens junctions (AJs) and desmosomes (DSs) (Farquhar and Palade, 1963). These junctional structures are typically aligned from the apical to the basal side, although DSs are independently distributed in other areas (Farquhar and Palade, 1963). Of these junctional structures, AJs play an essential role in physically connecting adjacent cells to maintain cell-cell junctions. At AJs, E-cadherin is a key Ca^{2+}-dependent cell-cell adhesion molecule (CAM) (Takeichi, 1988). E-cadherin first forms cis-dimers, which then join with other cis-dimers on adjacent cells to form trans-dimers, causing cell-cell adhesion. The cytoplasmic tail of E-cadherin is linked to the actin cytoskeleton through many peripheral membrane proteins, including α-catenin, β-catenin, vinculin, and α-actinin, which strengthen the cell-cell adhesion activity of E-cadherin (Gumbiner, 2005).

We recently discovered a new cell-cell adhesion system composed of nectins and afadin (encoded by *Mllt4*). These molecules colocalize with components of the cadherin-catenin system at AJs in many cell types, including epithelial cells and fibroblasts (Takai and Nakanishi, 2003). Nectins are Ca^{2+}-independent immunoglobulin (Ig)-like CAMs comprising a family of four members: nectin-1, nectin-2, nectin-3 and nectin-4 (Pvrl1-Pvrl4, respectively) (Takai and Nakanishi, 2003). Each nectin has one extracellular region with three Ig-like loops, one transmembrane segment and one cytoplasmic region. The cytoplasmic region of nectin is associated with the actin cytoskeleton through afadin, which is a nectin- and actin-filament (F-actin)-binding protein, whereas cadherin is associated with the actin cytoskeleton through many peripheral membrane proteins, including α- and β-catenins (Takai et al., 2003; Takai and Nakanishi, 2003; Takeichi, 1988). The extracellular regions of nectins associate with integrin $\alpha_v\beta_3$ (Sakamoto et al., 2006). During the formation of cell-cell junctions, the trans-interaction of nectins first occurs at the initial cell-cell contact sites, and then promotes the formation of cadherin-based AJs and the subsequent formation of claudin-based TJs (Yamada et al., 2006). This nectin-based cell-cell adhesion induces activation of Rap1, Cdc42 and Rac small G proteins through Src; this activation is necessary for the formation of cadherin-based AJs and claudin-based TJs (Fukuhara et al., 2004; Fukuyama et al., 2005; Kawakatsu et al., 2005; Kawakatsu et al., 2002; Shimizu and Takai, 2003). This nectin-induced signaling requires the interaction of nectin with integrin $\alpha_v\beta_3$, and the integrin-induced activation of protein kinase C and FAK (Ozaki et al., 2007; Sakamoto et al., 2006). Thus, the nectin-afadin complex plays a pivotal role in the formation of the junctional complex (Ogita and Takai, 2006; Takai and Nakanishi, 2003).

Although knockout mice of nectin-1, nectin-2 or nectin-3 were viable, these mice exhibited serious complications in several tissues, such as the brain, the testis, the epidermis and the ciliary body of the eye, because of the disorder of cell-cell adhesion caused by the loss of nectin (Honda et al., 2006; Inagaki et al., 2006; Inagaki et al., 2005; Ozaki-Kuroda et al., 2002; Wakamatsu

et al., 2007). Some functional redundancy might exist in each of the nectin-null mice, because many tissues express multiple nectins (Ogita and Takai, 2006; Takai and Nakanishi, 2003). By contrast, the disruption of afadin caused embryonic lethality in mice due to disorganization of the ectoderm, impaired migration of the mesoderm, and loss of somites and other structures derived from the ectoderm and mesoderm at stages during and after gastrulation of embryonic development (Ikeda et al., 1999). Cell-cell junctions, including AJs and TJs, were improperly formed in both the ectoderm and endoderm of afadin$^{-/-}$ embryos and embryonic bodies (EBs) (Komura et al., 2008). However, it remains unknown whether the disruption of afadin affects apoptosis, which plays a key role in embryogenesis (Penaloza et al., 2006).

In the present study, we first examined this issue using afadin$^{-/-}$ EBs, and found that afadin prevented apoptosis and was involved in cell survival. We then studied the molecular mechanism by which afadin is involved in cell survival using NIH3T3 cells as a model cell line. Growth factors, including PDGF, and their cognate receptors positively regulate cell survival by the activation of intracellular signaling – especially the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway, which blocks apoptotic signals and thus decreases the appearance of apoptotic cells (Burgering and Coffer, 1995; Downward, 2004; Franke et al., 1995). Thus, we examined whether afadin regulates PDGF-induced PI3K-Akt signaling and revealed the involvement of both nectin and afadin in the regulation of this signaling pathway. Several studies have shown that growth-factor receptors associate with CAMs such as cadherin to efficiently transduce the signals induced by growth factors into the intracellular signaling system (Comoglio et al., 2003; Perez-Moreno et al., 2003; Yap and Kovacs, 2003). In endothelial cells, the vascular endothelial growth factor (VEGF)-induced activation of PI3K and Akt through the VEGF receptor is supported by the VE-cadherin–β-catenin cell junctional complex, resulting in endothelial cell survival and angiogenesis (Carmeliet et al., 1999). We also examined whether nectin and/or afadin actually protect against apoptosis mediated by intrinsic and extrinsic pathways. Finally, we examined whether nectin and/or afadin associate with the PDGF receptor. Based on the results of these experiments, we demonstrate in this manuscript the roles of the nectin-afadin complex in cell survival.

Results

Inhibitory effect of afadin on apoptosis in EBs

To assess the effect of the nectin-afadin complex on cell survival, we first used EBs derived from wild-type and afadin$^{-/-}$ embryonic stem (ES) cells. As described previously (Ikeda et al., 1999; Komura et al., 2008), EBs derived from both wild-type and afadin$^{-/-}$ ES cells had a two-layered cystic structure with outer endodermal and inner ectodermal layers. EBs from suspension culture were fixed every other day from day 9 to day 17. From these, we observed that the central cavity of EBs derived from afadin$^{-/-}$ ES cells was full of apoptotic cells, which were detected by the terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end-labeling (TUNEL) method and by immunohistochemistry using the cleaved caspase-3 monoclonal antibody (mAb), whereas the cavity of EBs derived from wild-type ES cells was almost empty and hardly contained any TUNEL- or cleaved-caspase-3-positive cells at every time point observed (Fig. 1 and data not shown). These results indicate that afadin prevents apoptosis and is involved in cell survival during embryonic development.

Involvement of afadin in PDGF-induced Akt activation and cell survival in NIH3T3 cells

To explore the molecular mechanisms underlying the role of afadin in cell survival, we generated NIH3T3 cells in which afadin was stably knocked down (afadin-knockdown NIH3T3 cells), and examined the effect of afadin on the phosphorylation of Akt, which is crucially involved in anti-apoptotic pathways (Downward, 2004). When control NIH3T3 cells were treated with PDGF for the indicated periods of time (Fig. 2A), the phosphorylation of Akt was increased in a time-dependent manner. However, this increase in the phosphorylation of Akt was significantly suppressed in afadin-knockdown NIH3T3 cells. By contrast, PDGF-induced phosphorylation of PDGF receptor and Src, an intracellular signaling molecule that is also known to be activated in response to PDGF (DeMali et al., 1999), was not affected by knockdown of afadin. These results indicate that afadin regulates the PDGF-induced phosphorylation of Akt independently of the activation of PDGF receptor and other intracellular signaling molecules such as Src. However, given that the inhibition of Akt phosphorylation by knockdown of afadin was not complete, other signaling molecules might additionally participate in the regulation of cell-survival signaling in NIH3T3 cells.

We then examined whether knockdown of afadin actually enhances apoptosis of NIH3T3 cells via both intrinsic and extrinsic pathways. The intrinsic pathway is activated in response to stress, such as nutrient deprivation, whereas the extrinsic pathway is induced by receptor binding to pro-apoptotic death ligands such as Fas ligand (Jin and El-Deiry, 2005). When confluent NIH3T3 cells were cultured in medium without serum, the number of apoptotic cells detected by the TUNEL method was increased by knockdown of afadin (Fig. 2B). We also examined whether apoptosis induced by serum deprivation is prevented by growth factors, such as PDGF and epidermal growth factor (EGF), because these growth factors are well known to play key roles in cell survival (Burgering and Coffer, 1995; Franke et al., 1995; Henson and Gibson, 2006). In the presence of PDGF, the number of apoptotic cells was reduced in control NIH3T3 cells, but not in afadin-knockdown cells, whereas in the presence of EGF, apoptosis was suppressed in both control and afadin-knockdown cells, suggesting a significant relationship between afadin and the PDGF receptor anti-apoptotic pathways. Essentially the same results were obtained when cells were stimulated with Fas ligand (Fig. 2C). The amount of cleaved caspase-3 produced by serum starvation or Fas-ligand stimulation was detected by western blotting (Fig. 2D) and was consistent with the results obtained with the TUNEL method, as shown in Fig. 2B,C. Taken together, these results indicate that afadin prevents apoptosis in both the intrinsic and extrinsic pathways and positively regulates cell survival, preferentially utilizing PDGF-initiated and Akt-mediated signaling.

We also confirmed that the activation of Akt itself exerted anti-apoptotic effects. After stimulation with Fas ligand, the number of cleaved-caspase-3-positive cells determined by immunofluorescence experiments and the amount of cleaved caspase-3 detected by western blotting were significantly reduced in afadin-knockdown NIH3T3 cells stably expressing a GFP-tagged constitutively active mutant of Akt (Akt-CA-GFP), compared with afadin-knockdown NIH3T3 cells stably expressing GFP (Fig. 2E,F).

Significance of the binding of afadin to nectin for afadin-mediated anti-apoptotic signaling

We further examined whether the re-expression of afadin in afadin-knockdown NIH3T3 cells rescues the reduced phosphorylation of

Akt. When GFP-tagged full-length afadin (GFP-afadin), which was resistant to RNAi against afadin, was stably expressed in afadin-knockdown NIH3T3 cells, the level of phosphorylated Akt was increased similar to that observed in control NIH3T3 cells after treatment with PDGF (Fig. 3A). However, when the RNAi-resistant GFP-tagged PDZ-domain-lacking afadin mutant (GFP-afadinΔPDZ) was stably expressed in afadin-knockdown NIH3T3 cells, the reduced phosphorylation of Akt was not rescued, although the expression level of GFP-afadinΔPDZ was almost equal to that of full-length GFP-afadin. Because afadin directly interacts with nectin through the PDZ domain (Takahashi et al., 1999), these results indicate that the binding of afadin to nectin is important for the regulation of Akt activation.

Consistent with these results, after stimulation with Fas ligand the number of apoptotic afadin-knockdown NIH3T3 cells re-expressing GFP-afadin was lower than the number of apoptotic afadin-knockdown NIH3T3 cells stably expressing GFP and was similar to the number of apoptotic control NIH3T3 cells stably expressing GFP (Fig. 3B). However, stable re-expression of GFP-afadinΔPDZ did not reduce the number of apoptotic cells. The amount of cleaved caspase-3 was also reduced by re-expression of GFP-afadin, but not of GFP-afadinΔPDZ (Fig. 3C).

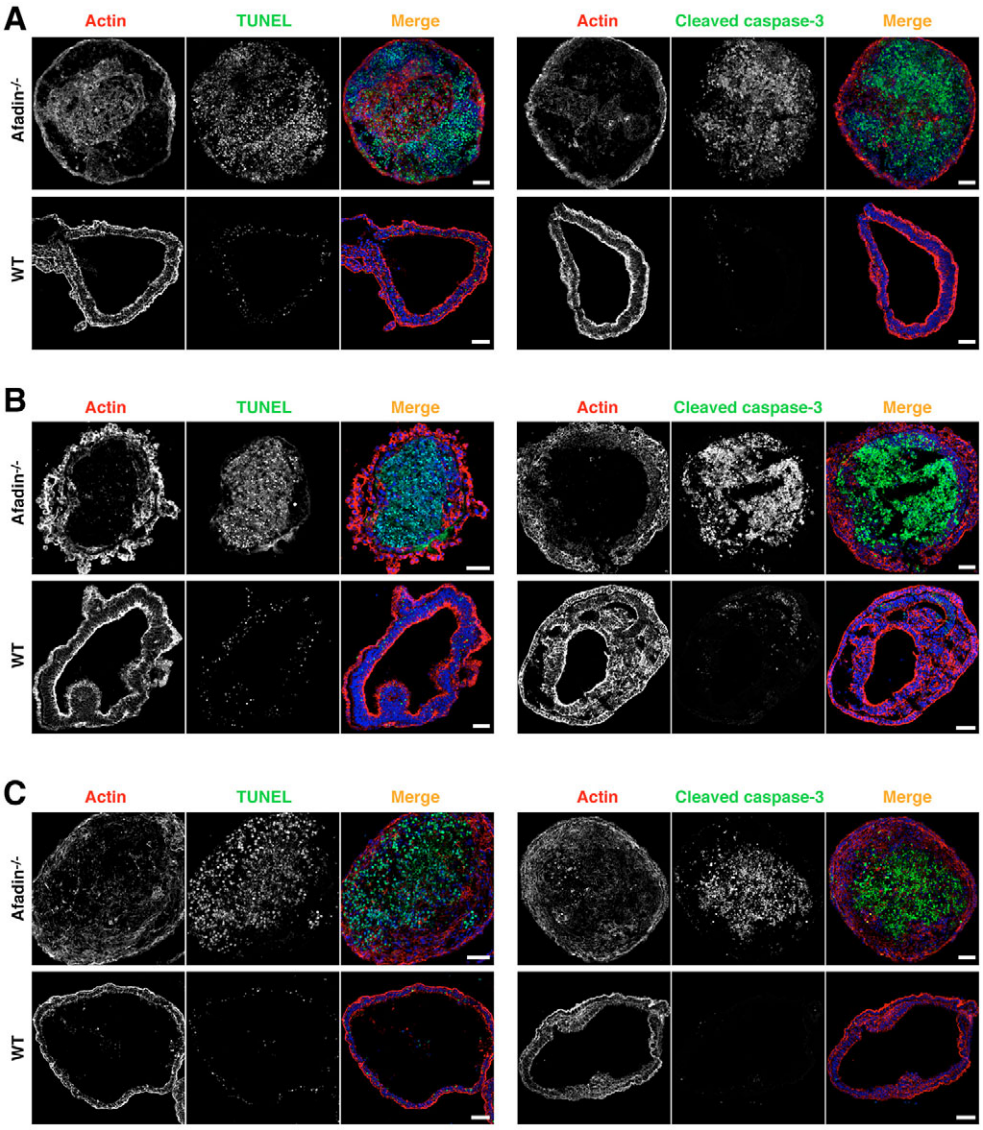

Fig. 1. Enhancement of apoptosis in EBs derived from afadin$^{-/-}$ ES cells. EBs formed from wild-type and afadin$^{-/-}$ ES cells were fixed, and apoptotic cells were detected using the TUNEL method and immunostaining with the anti-cleaved-caspase-3 mAb. F-actin (red) and nuclei (blue) were counterstained with rhodamine-phalloidin and DAPI, respectively. (A) EBs at 9 days after the withdrawal of leukemia inhibitory factor (LIF). (B) EBs at 11 days after the withdrawal of LIF. (C) EBs at 15 days after the withdrawal of LIF. Scale bars: 50 μm. The results shown in this figure are representative of three independent experiments.

Involvement of nectin-3 in PDGF-induced cell survival in NIH3T3 cells

We next investigated the effect of nectin on the PDGF-induced phosphorylation of Akt, because the above results strongly suggested the significance of the binding of afadin to nectin in this signaling pathway. We knocked down nectin-3 in NIH3T3 cells by transient transfection with an siRNA against nectin-3 (nectin-3-knockdown NIH3T3 cells) and confirmed that the knockdown of nectin-3 did not affect the expression level of afadin in NIH3T3 cells (supplementary material Fig. S1). By contrast, the expression levels of nectin-1 and nectin-3 in afadin-knockdown NIH3T3 cells were almost equal to those in control cells, although the level of nectin-3 expression was slightly lower and the slowest migrating isoform of nectin-1 disappeared in afadin-knockdown NIH3T3 cells. Although the exact reason for these changes is unclear, afadin might be somewhat involved in the stability of nectin, because afadin contributes to the connection of nectin to the actin cytoskeleton and its stabilization on the cell surface at AJs. When control or nectin-3-knockdown NIH3T3 cells were treated with PDGF for the indicated periods of time (Fig. 4A), the level of phosphorylation of Akt was significantly reduced in nectin-3-knockdown NIH3T3 cells compared with control NIH3T3 cells, whereas the levels of phosphorylation of PDGF receptor and Src in nectin-3-knockdown NIH3T3 cells were equal to those in control NIH3T3 cells (Fig. 4A). These results were similar to those seen in afadin-knockdown NIH3T3 cells. In addition, knockdown of nectin-3 increased the number of apoptotic cells, just as knockdown of afadin did (Fig. 4B). These results indicate that, similar to afadin, nectin-3 regulates PDGF-induced Akt signaling and has an anti-apoptotic effect in NIH3T3 cells.

Furthermore, the reduced phosphorylation of Akt in nectin-3-knockdown NIH3T3 cells was rescued by transient re-expression of FLAG-tagged full-length nectin-3 in these cells, but not by

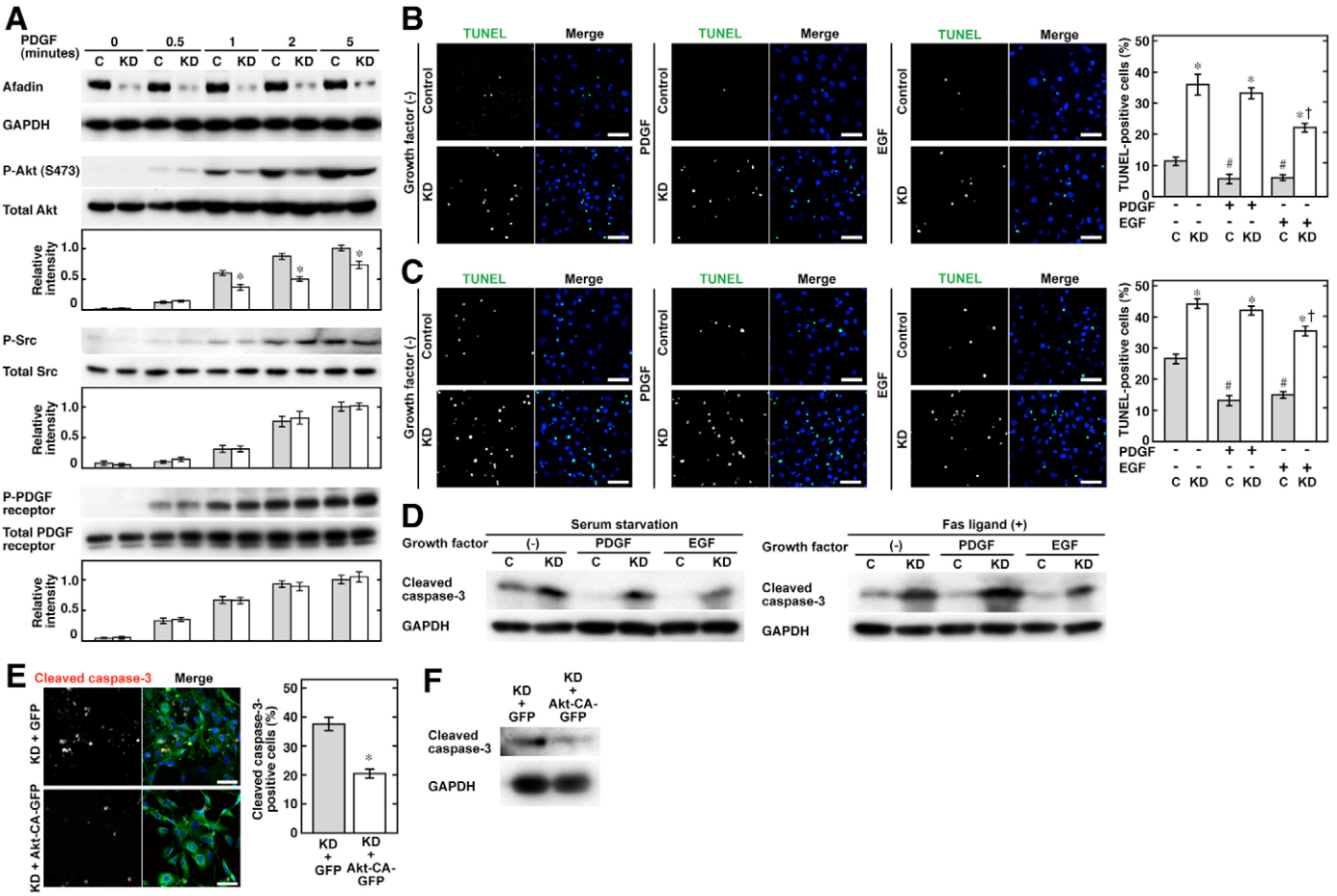

Fig. 2. Involvement of afadin in PDGF-induced Akt activation and cell survival in NIH3T3 cells. (A) Inhibition of the PDGF-induced phosphorylation of Akt by knockdown of afadin in NIH3T3 cells. After 16 hours of serum starvation, control (denoted by C) and afadin-knockdown (KD) NIH3T3 cells were treated with 3 ng/ml PDGF for the indicated periods of time. Cell lysates were subjected to western blotting with the indicated antibodies. GAPDH was immunoblotted as a loading control. Bars in the graph under each blot represent the relative band intensities of phosphorylated Akt (top), Src (middle) and PDGF receptor (bottom) normalized to the total amount of each protein as compared with the values from control cells treated with PDGF for 5 minutes, which are expressed as 1. Grey bars, control cells; white bars, KD cells. Error bars indicate s.e.m. *P<0.05 vs control NIH3T3 cells. (B) Increase in serum-starvation-induced apoptosis by knockdown of afadin in NIH3T3 cells. Confluent control and afadin-knockdown NIH3T3 cells were serum-starved for 16 hours and then treated with or without 3 ng/ml PDGF or 3 ng/ml EGF for 24 hours. Apoptotic cells were detected using the TUNEL method. Nuclei were counterstained with DAPI and are seen in blue. Bars in the graph to the right represent the mean percentages of TUNEL-positive cells in three independent experiments. In each experiment, the number of TUNEL-positive cells among a total of 100 cells observed in four different fields of view was counted. Error bars indicate s.e.m. *P<0.05 vs paired control cells; $^{\#}P$<0.05 vs control cells without growth-factor treatment; $^{\dagger}P$<0.05 vs afadin-knockdown cells without growth-factor treatment. (C) Increase in Fas-ligand-induced apoptosis by knockdown of afadin in NIH3T3 cells. Confluent control and afadin-knockdown NIH3T3 cells were serum-starved for 16 hours and then stimulated with 100 ng/ml Fas ligand (Jo2 mAb) concomitant with or without 3 ng/ml PDGF or 3 ng/ml EGF for 24 hours. Apoptotic cells were counted as described in B. Error bars indicate s.e.m. and symbols indicate the same as shown in B. (D) Increased production of cleaved caspase-3 by knockdown of afadin in NIH3T3 cells. Confluent control and afadin-knockdown NIH3T3 cells were cultured in the same conditions described in B and C. Cell lysates were subjected to western blotting with the anti-cleaved-caspase-3 pAb. GAPDH was immunoblotted as a loading control. (E) Inhibitory effect of Akt-CA on apoptosis in afadin-knockdown NIH3T3 cells. Confluent afadin-knockdown NIH3T3 cells transfected with GFP or Akt-CA-GFP were serum-starved for 16 hours and then stimulated with 100 ng/ml Fas ligand for 24 hours in the presence of 3 ng/ml PDGF. Cells were immunostained with the anti-cleaved-caspase-3 mAb and nuclei were counterstained with DAPI. The graph represents the mean percentage of cleaved-caspase-3-positive cells assessed as described in B. Error bars indicate s.e.m. *P<0.05 vs control NIH3T3 cells. (F) Reduced production of cleaved caspase-3 by the expression of Akt-CA in afadin-knockdown NIH3T3 cells. Cell lysates from control and afadin-knockdown NIH3T3 cells cultured in the same conditions as described in E were subjected to western blotting with the anti-cleaved-caspase-3 pAb. GAPDH was immunoblotted as a loading control. Scale bars: 50 μm.

transient re-expression of FLAG-nectin-3ΔC, which lacks the C-terminal four amino acids necessary for the binding of afadin (Fig. 4C). This provides supportive evidence that the binding of afadin to nectin is necessary for PDGF-induced Akt activation.

Regulation of PI3K activity by the nectin-afadin complex in NIH3T3 cells

We then examined at which step nectin and afadin affect the PDGF-initiated signaling cascade leading to Akt phosphorylation. Because the level of phosphorylation of PDGF receptor induced by PDGF stimulation was not different between nectin-3- or afadin-knockdown NIH3T3 cells and control NIH3T3 cells, we assumed that nectin and afadin would exert their effects downstream of PDGF receptor and upstream of Akt. PI3K is well-known to bind to phosphorylated PDGF receptor and to function directly downstream of PDGF receptor and upstream of Akt (Franke et al., 1995; Hu et al., 1992). Thus, nectin and afadin are likely to regulate the activation of PI3K. To confirm this, we measured the kinase activity

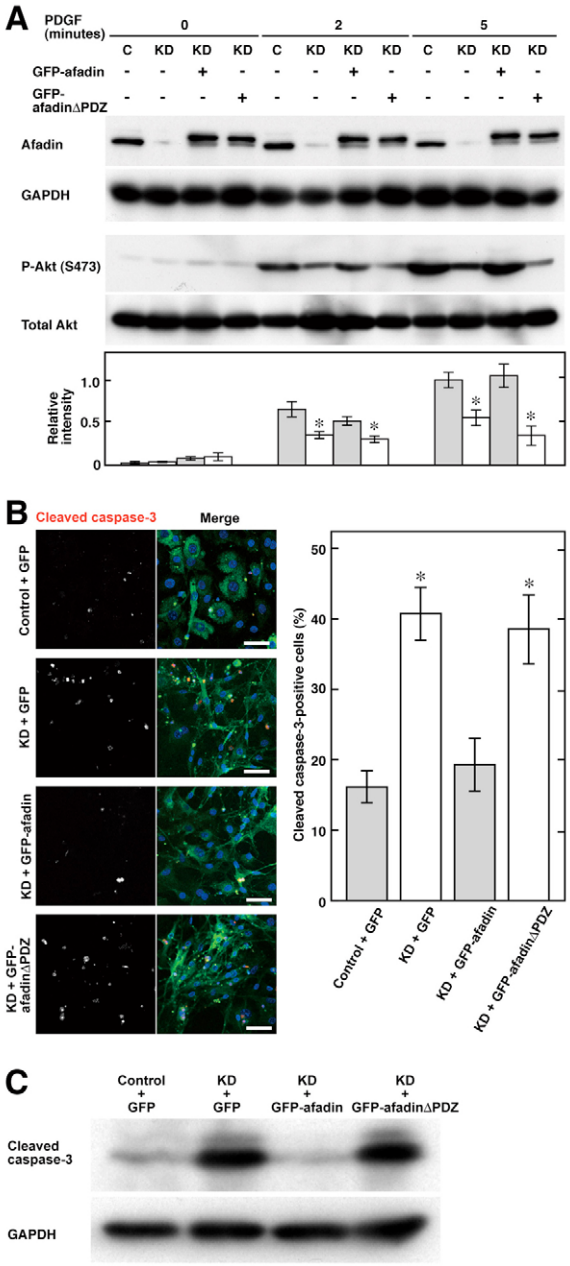

Fig. 3. Significance of the binding of afadin to nectin for the prevention of apoptosis. (A) Restoration of PDGF-induced phosphorylation of Akt by re-expression of full-length afadin, but not afadinΔPDZ, in afadin-knockdown NIH3T3 cells. Four types of cells (control NIH3T3 cells, afadin-knockdown NIH3T3 cells, afadin-knockdown NIH3T3 cells re-expressing GFP-tagged full-length afadin and afadin-knockdown NIH3T3 cells re-expressing GFP-tagged afadinΔPDZ) were treated with PDGF for the indicated periods of time. Cell lysates were subjected to western blotting with the indicated antibodies. GAPDH was immunoblotted as a loading control. The relative band intensity was calculated as described in Fig. 2A. **P*<0.05 vs control NIH3T3 cells. C, control; KD, afadin knockdown. (B) Reduction of apoptosis by re-expression of full-length afadin in afadin-knockdown NIH3T3 cells. Confluent control and afadin-knockdown NIH3T3 cells stably expressing GFP, GFP-afadin or GFP-afadinΔPDZ were serum-starved for 16 hours and then stimulated with 100 ng/ml Fas ligand for 24 hours with 3 ng/ml PDGF. Cells were immunostained with the anti-cleaved-caspase-3 mAb (red) and nuclei were counterstained with DAPI (blue). The graph represents the mean percentage of cleaved-caspase-3-positive cells in three independent experiments. In each experiment, the number of cleaved-caspase-3-positive cells among a total of 100 cells observed in four different fields of view was counted. Error bars indicate s.e.m. **P*<0.05 vs control NIH3T3 cells expressing GFP. Scale bars: 50 μm. (C) Reduced expression of cleaved caspase-3 by re-expression of full-length afadin in afadin-knockdown NIH3T3 cells. Control NIH3T3 cells, afadin-knockdown NIH3T3 cells, afadin-knockdown NIH3T3 cells re-expressing GFP-tagged full-length afadin and afadin-knockdown NIH3T3 cells re-expressing GFP-tagged afadinΔPDZ were cultured in the same conditions as described in B. Lysates from these cells were subjected to western blotting with the anti-cleaved-caspase-3 pAb. GAPDH was immunoblotted as a loading control.

of PI3K in control, nectin-3-knockdown and afadin-knockdown NIH3T3 cells before and after treatment with PDGF. Before PDGF treatment, the kinase activity of PI3K was low and similar among all types of cells used in this assay. However, after PDGF treatment, the kinase activity in nectin-3-knockdown and afadin-knockdown NIH3T3 cells was almost abrogated (Fig. 5A).

To explore the association of afadin with PI3K, we performed an immunoprecipitation assay. When FLAG-afadin was transfected into HEK293 cells it was co-immunoprecipitated with the PI3K subunit p85 (Fig. 5B). We also confirmed the co-immunoprecipitation of endogenous afadin with p85 in NIH3T3 cells (Fig. 5C). These results indicate that the nectin-afadin complex regulates PDGF-induced Akt signaling at the step of PI3K activation through the association of afadin with PI3K. However, we did not detect the direct binding of afadin to p85 by affinity chromatography using pure recombinant proteins (data not shown), suggesting that post-translational modification(s) of either afadin, p85, or both, and/or an unidentified molecule(s) are involved in the binding of afadin with p85.

Association of the nectin-afadin complex with PDGF receptor

In the last set of experiments, we examined the association of the nectin-afadin complex with PDGF receptor. Nectin-3 and afadin colocalized with PDGF receptor at cell-cell boundaries in confluent NIH3T3 cells (Fig. 6Aa and supplementary material Fig. S2). However, this colocalization was not observed in nectin-3-knockdown NIH3T3 cells, although afadin was retained at cell-cell adhesion sites (Fig. 6Ab), suggesting the physical association of nectin-3, but not afadin, with PDGF receptor. Consistent with this, the association of afadin with PDGF receptor was not detected in co-immunoprecipitation assays (data not shown). The reason for the retention of afadin at cell-cell adhesion sites in nectin-3-knockdown cells is because of the fact that other nectins, such as nectin-1, also exist in NIH3T3 cells. Because the expression level of PDGF receptor was unchanged by knockdown of nectin-3 (Fig. 4A), PDGF receptor is assumed to be diffusely distributed on the cell surface of nectin-3-knockdown NIH3T3 cells.

We then confirmed the physical association of nectin with PDGF receptor in bead-cell-contact and co-immunoprecipitation assays. In the bead-cell-contact assay, NIH3T3 cells were incubated with microbeads coated with Nef-1, which is the extracellular region of nectin-1 fused to IgG Fc. Out of all the nectin family members, nectin-1 and nectin-3 trans-interact most strongly (Honda et al., 2003a). The immunofluorescence signal for nectin-3 was concentrated at the contact sites between the Nef-1-coated beads and NIH3T3 cells, as previously described (Honda et al., 2003b; Sakamoto et al., 2006), and the signal for PDGF receptor was also concentrated there (Fig. 6Ba). By contrast, when NIH3T3 cells were

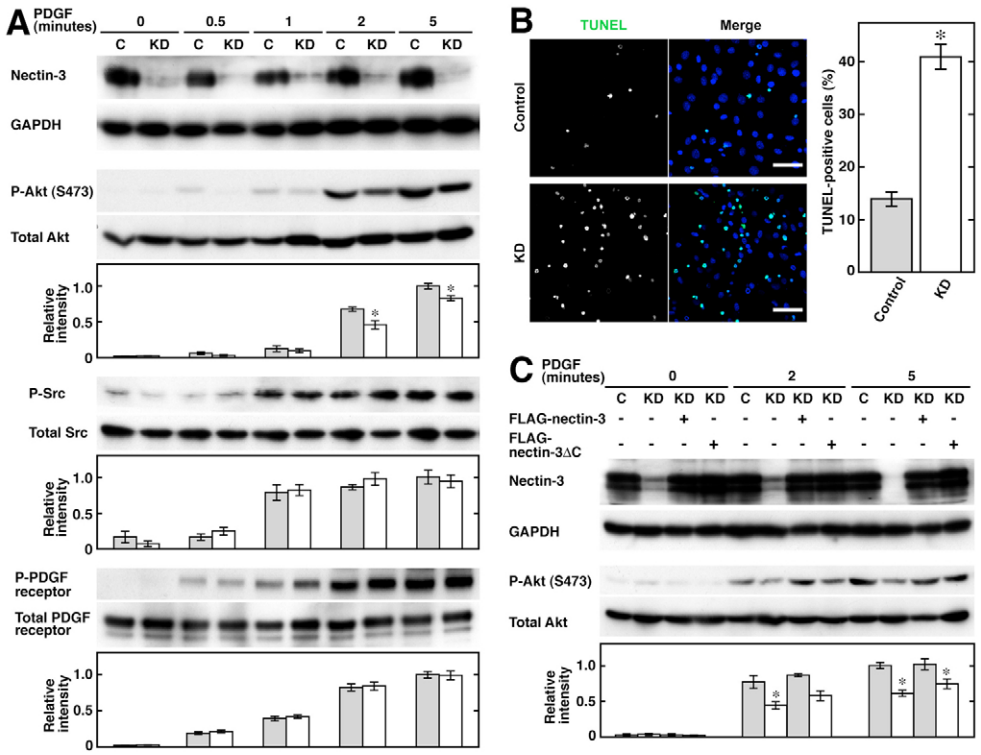

Fig. 4. Involvement of nectin-3 in PDGF-induced phosphorylation of Akt and cell survival in NIH3T3 cells. (A) Inhibition of PDGF-induced phosphorylation of Akt by knockdown of nectin-3 in NIH3T3 cells. Confluent control and nectin-3-knockdown NIH3T3 cells were treated for the indicated periods of time. Cell lysates were subjected to western blotting with the indicated antibodies. GAPDH was immunoblotted as a loading control. The relative band intensity was calculated as described in Fig. 2A. **P*<0.05 vs control NIH3T3 cells. (B) Increased number of apoptotic nectin-3-knockdown NIH3T3 cells. Confluent control and nectin-3-knockdown NIH3T3 cells were serum-starved for 16 hours and then stimulated with 100 ng/ml Fas ligand for 24 hours in the presence of 3 ng/ml PDGF. Apoptotic cells were detected using the TUNEL method. Nuclei were counterstained with DAPI and are seen in blue. The graph represents the mean percentage of TUNEL-positive cells assessed as described in Fig. 2C. **P*<0.05 vs control NIH3T3 cells. Scale bars: 50 μm. (C) Requirement for the association of nectin-3 with afadin in order to obtain PDGF-induced phosphorylation of Akt. Four types of cells (control NIH3T3 cells, nectin-3-knockdown NIH3T3 cells, nectin-3-knockdown NIH3T3 cells rescued by FLAG-tagged full-length nectin-3 and nectin-3-knockdown NIH3T3 cells rescued by FLAG-tagged nectin-3 lacking the C-terminal four amino acids) were treated with PDGF for the indicated periods of time. Cell lysates were subjected to western blotting with the indicated antibodies. GAPDH was immunoblotted as a loading control. The relative band intensity was calculated as described in Fig. 2A. **P*<0.05 vs control NIH3T3 cells. C, control; KD, nectin-3 knockdown.

incubated with concanavalin A (ConA)-coated microbeads as a control, neither nectin-3 nor PDGF receptor were concentrated at the bead-cell contact sites (Fig. 6Bb). In the co-immunoprecipitation assay, endogenous PDGF receptor and nectin-3 were co-immunoprecipitated in NIH3T3 cells (Fig. 6C).

We further examined whether PDGF receptor associates with other nectins besides nectin-3. When PDGF receptor was transiently co-expressed with FLAG-tagged nectin-1, nectin-2, nectin-3 or nectin-4 in HEK293 cells and cell lysates were immunoprecipitated with the anti-FLAG mAb, PDGF receptor was only co-immunoprecipitated with FLAG–nectin-3 (Fig. 6D). Consistent with this result, the transient knockdown of nectin-1 in NIH3T3 cells did not increase the number of apoptotic cells (supplementary material Fig. S3). In addition, EGF receptor, another growth-factor receptor, was not co-immunoprecipitated with nectin-3 (Fig. 6E), indicating that the association of PDGF receptor with nectin-3 is unlikely to be unspecific.

We examined in more detail which regions of PDGF receptor and nectin-3 are involved in the association of these molecules. When the co-immunoprecipitation assay was carried out in HEK293 cells transiently expressing PDGF receptor with either a FLAG-tagged cytoplasmic-region-truncated (ΔCP) or FLAG-tagged extracellular-region-truncated (ΔEC) form of nectin-3, PDGF receptor was co-immunoprecipitated with FLAG–nectin-3ΔCP, but not with FLAG–nectin-3ΔEC (Fig. 6F), indicating the necessity of the extracellular region of nectin-3 for its association with PDGF receptor. Furthermore, the association between PDGF receptor and nectin-3 was independent of receptor phosphorylation (Fig. 6G). Collectively, these results indicate that nectin-3 physically associates with PDGF receptor through their respective extracellular regions, irrespective of the PDGF-induced phosphorylation of PDGF receptor.

Discussion

In this study, we first demonstrated the protective effect of afadin against apoptosis during early embryogenesis, using EBs as a model system. Similarly, such a protective effect of afadin was also observed in cultured NIH3T3 cells when apoptosis was induced by serum starvation or Fas-ligand stimulation, indicating the involvement of afadin in the inhibition of both intrinsic and extrinsic pathways of apoptosis. We also revealed that afadin and its binding partner nectin, one of the cell-cell adhesion molecules at AJs, play a pivotal role in the positive regulation of PDGF-induced activation of the PI3K-Akt signaling, which mediates cell survival by enhancing the kinase activity of PI3K. The formation of a complex containing nectin and afadin is necessary for this regulation. Although the roles of nectin and afadin in the formation of AJs and TJs have been extensively studied (Ogita and Takai, 2006; Takai and Nakanishi, 2003), little is known about the roles of these molecules in cell survival. The findings presented in this study provide a new insight into the molecular mechanisms of cell survival mediated by the nectin-afadin complex, a known cell-adhesion system.

There have been some reports describing the involvement of another cell-cell adhesion molecule, cadherin, in growth-factor-induced intracellular signaling (Comoglio et al., 2003; Perez-Moreno et al., 2003; Yap and Kovacs, 2003). In addition to previous reports, our data clearly indicate that the nectin-afadin complex crosstalks with the PDGF-receptor-mediated signaling pathway in multiple aspects; on the cell surface, PDGF receptor itself interacts with nectin-3, whereas inside the cell, the PDGF-

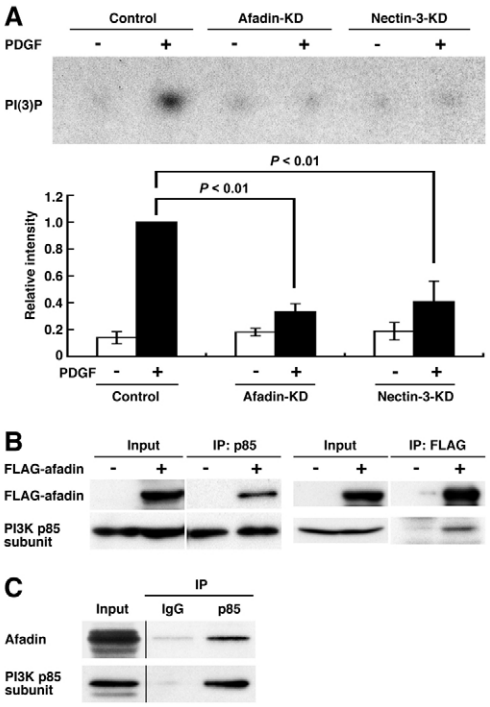

Fig. 5. Nectin-3- and afadin-mediated regulation of PI3K activity in NIH3T3 cells. (A) Inhibition of the PDGF-induced increase in PI3K activity by knockdown of nectin-3 and afadin in NIH3T3 cells. After 16 hours of serum starvation, control, nectin-3-knockdown and afadin-knockdown NIH3T3 cells were treated with 3 ng/ml PDGF for 2 minutes. Lysates from cells harvested before and after PDGF treatment were subjected to an assay for PI3K activity. Bars in the graph represent the relative spot intensity compared with the values of control NIH3T3 cells treated with PDGF, which are expressed as 1. KD, knockdown; PI(3)P, phosphatidylinositol 3-phosphate. (B) Association of FLAG-afadin with PI3K. Untransfected HEK293 cells and HEK293 cells transiently transfected with FLAG-afadin were lysed and the cell lysates were immunoprecipitated with the anti-FLAG mAb or the anti-p85 pAb. The immunoprecipitates were subjected to western blotting with the anti-p85 pAb and the anti-FLAG mAb. (C) Association of endogenous afadin and PI3K in NIH3T3 cells. Lysates of NIH3T3 cells were immunoprecipitated with the anti-p85 pAb or non-immunized rabbit IgG as a control, and the immunoprecipitates were subjected to western blotting with the anti-afadin mAb and the anti-p85 pAb. The results shown in this figure are representative of three independent experiments.

receptor-binding molecule p85, a subunit of PI3K, interacts with afadin (Fig. 7). These versatile and harmonious crosstalks are essential for the prevention of apoptosis and enhancement of cell survival in confluent cells, because the depletion of either afadin or nectin-3 alone significantly affected PDGF-induced PI3K-Akt signaling and increased apoptosis. Thus, afadin is likely to exert an anti-apoptotic effect in the context of its binding to nectin-3. By contrast, we recently showed that nectin associates with the cell-matrix adhesion molecule integrin $\alpha_v\beta_3$; we also showed that these two different types of cell-adhesion molecules cooperatively promote the nectin-induced formation of AJs and crosstalk with each other during the formation of AJs (Sakamoto et al., 2006; Sakamoto et al., 2008). It is possible that nectin, integrin $\alpha_v\beta_3$ and PDGF receptor form a ternary complex at AJs, but further investigations are required to verify the possibility and significance of this ternary complex.

In the present study, knockdown of afadin or nectin-3 caused relatively rapid suppression of the PDGF-induced phosphorylation of Akt, taking only a few minutes. However, the effect of knockdown of afadin or nectin-3 on the entire process of apoptosis, as detected by TUNEL analysis, usually took a day to become apparent. These results raise the issue as to why there is such a time lag between the apoptosis-related signaling and the execution of apoptosis. Recent results have demonstrated that the pro-apoptotic-stimuli-induced reduction of mitochondrial functions, usually observed at the initial step of apoptosis, takes a few minutes, and that this event seems to irreversibly lead to apoptosis-associated cell death within hours (Green, 2005). Thus, inhibition of PDGF-induced Akt phosphorylation in a relatively short period by knockdown of afadin or nectin-3 would be significant for the consequent process of apoptosis.

Although knockdown of afadin or nectin-3 significantly inhibited PDGF-induced phosphorylation of Akt, an increase in the phosphorylation of Akt occurred in afadin- or nectin-3-knockdown NIH3T3 cells after treatment with PDGF. The exact reason why such residual phosphorylation of Akt in these cells is not enough to prevent apoptosis remains unclear, but any difference in the amount of Akt phosphorylation between control and afadin- or nectin-3-knockdown NIH3T3 cells would be amplified in the downstream signaling pathway mediating apoptosis, so that this difference might crucially contribute to the differential regulation of molecules in this pathway, such as GSK3, a protein substrate of Akt that is inactivated by Akt-dependent phosphorylation (Cohen and Frame, 2001). A recent study showed an important role of GSK3 in the regulation of apoptosis through MCL1 (Maurer et al., 2006). Additional studies might be necessary to completely address this issue in the future.

Materials and Methods

Expression vectors and chemical reagents

The human PDGFβ-receptor cDNA (GenBank accession number BC032224) was kindly provided by T. Matsui (Kobe University, Kobe, Japan) and was inserted into the pCMV5 vector (pCMV5-βPDGFR). Expression vectors for FLAG–nectin-1 (aa 27-518, pFLAG-CMV1-nectin-1), FLAG–nectin-2 (aa 30-467, pFLAG-CMV1-nectin-2), FLAG–nectin-3 (aa 56-549, pCAGIPuro-FLAG-nectin-3), FLAG–nectin-4 (aa 29-508, pFLAG-CMV1-nectin-4), FLAG–nectin-3ΔCP (aa 56-430, pFLAG-CMV1-nectin-3ΔCP) and FLAG–nectin-3ΔEC (aa 395-549, pFLAG-CMV1-nectin-3ΔEC) were constructed as previously described (Sakamoto et al., 2006). The accession numbers for the cDNAs of human nectin-1, mouse nectin-2, mouse nectin-3 and mouse nectin-4 are AF060231, M80206, AF195833 and BC024948, respectively. An expression vector for FLAG-afadin (pCMVF-afadin) was constructed by inserting full-length rat afadin cDNA (accession number U83230) into pCMVF vector, which was generously supplied by K. Matsumoto (Nagoya University, Nagoya, Japan). An expression vector for Akt-CA-GFP (pCAGIpuro-Akt-CA-GFP) was constructed by inserting Akt-CA cDNA, which was kindly provided by Y. Gotoh (Tokyo University, Tokyo, Japan; accession number for wild-type human Akt cDNA: BC000479), into pEGFP-N3 (Clontech) and then the cDNA fragment coding Akt-CA-GFP was cut out and ligated into the pCAGIpuro vector. A mammalian expression vector for EGF receptor (pME18S-EGFR; accession number for human EGF receptor cDNA: X00588) was kindly provided by T. Yamamoto (The University of Tokyo, Tokyo, Japan). Human PDGF-BB, EGF and AG1296 were purchased from Peprotech, Sigma and Calbiochem, respectively.

Antibodies

The mouse mAb against afadin was prepared as described previously (Sakisaka et al., 1999), and the rat mAb and rabbit pAb against nectin-3 and the rabbit pAb against nectin-1 were also prepared as described (Satoh-Horikawa et al., 2000; Takahashi et al., 1999). The following rabbit pAbs were purchased from commercial sources: anti-PDGF receptor (Santa Cruz Biotechnology, sc-432), anti-phospho-PDGF receptor (Tyr^{857}) (Santa Cruz Biotechnology, sc-12907-R), anti-Akt (Cell Signaling Technology, #9272), anti-phospho-Akt (Ser^{473}) (Cell Signaling Technology, #9271), anti-Src (Cell Signaling Technology, #2108), anti-phospho-Src (Tyr^{416}) (Cell Signaling Technology, #2101), anti-cleaved-caspase-3 (Cell Signaling Technology, #9661), and anti-PI3K (p85 subunit) (Upstate, #06-497). The mouse mAbs listed below were also purchased from commercial sources: anti-EGF receptor (clone 13; Pharmingen, #610017), anti-FLAG (M2; Sigma, F3165) and anti-GFP (JL-8; Clontech, #632380). The rabbit mAbs against PDGF receptor (Y92; ab32570) and cleaved caspase-3 (C92-

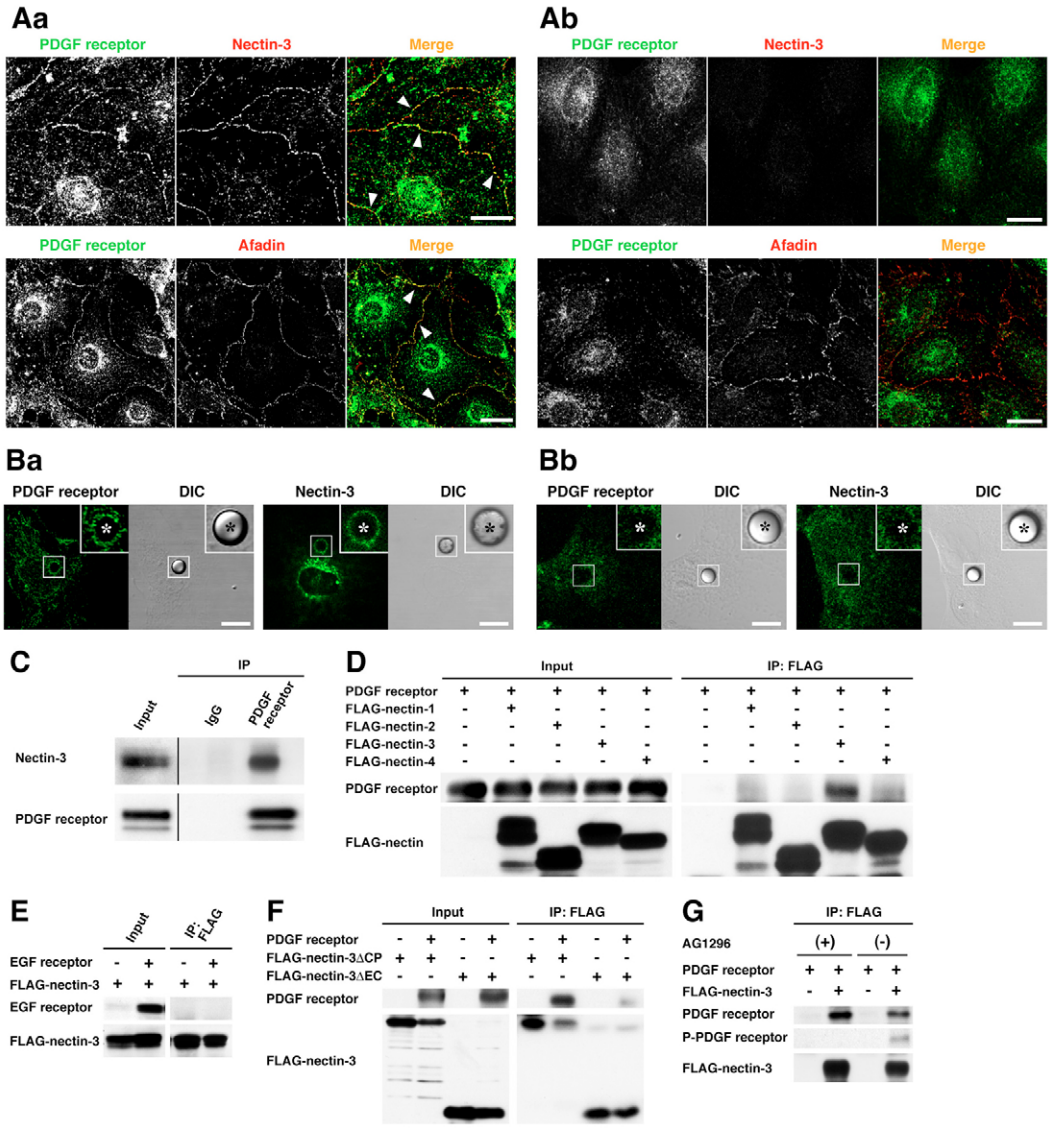

Fig. 6. Association of the nectin-afadin complex with PDGF receptor. (A) Colocalization of the nectin-afadin complex with PDGF receptor at cell-cell adhesion sites. Confluent NIH3T3 cells (Aa) or nectin-3-knockdown NIH3T3 cells (Ab) were immunostained with the anti-PDGF receptor pAb (Santa Cruz Biotechnology) and the rat anti-nectin-3 or mouse anti-afadin mAb. Arrowheads indicate the colocalization sites of nectin-3 or afadin with PDGF receptor. Scale bars: 10 µm. (B) Recruitment of nectin-3 and PDGF receptor at the contact sites between Nef-1-coated beads and NIH3T3 cells. NIH3T3 cells were incubated with Nef-1-coated microbeads (Ba) or ConA-coated microbeads (Bb) for 1 hour; they were then fixed and immunostained with the anti-PDGF receptor pAb and the anti-nectin-3 mAb. The positions of the beads are marked with asterisks. Insets are higher-magnified images of boxed areas. DIC, differential interference contrast. Scale bars: 10 µm. (C) Co-immunoprecipitation of endogenous PDGF receptor with nectin-3. The cell lysates of NIH3T3 cells treated with a chemical cross-linker DTSSP were immunoprecipitated with the anti-p85 pAb or normal rabbit IgG as a control, and were subjected to western blotting with the anti-nectin-3 pAb and the anti-PDGF receptor pAb. (D) Co-immunoprecipitation of PDGF receptor with nectin-3. Lysates of HEK293 cells transiently expressing PDGF receptor with or without FLAG-nectin molecules were immunoprecipitated with the anti-FLAG mAb. The immunoprecipitates were subjected to western blotting with the anti-PDGF receptor pAb and the anti-FLAG mAb. (E) No co-immunoprecipitation of EGF receptor with nectin-3 occurred. Lysates of HEK293 cells transiently expressing FLAG-nectin-3 with or without EGF receptor were immunoprecipitated with the anti-FLAG mAb, followed by western blotting as described in D. (F) Co-immunoprecipitation of PDGF receptor with nectin-3 through their extracellular regions. The cell lysates of HEK293 cells transiently expressing FLAG–nectin-3ΔCP or FLAG–nectin-3ΔEC with or without PDGF receptor were immunoprecipitated with the anti-FLAG mAb, followed by western blotting, as described in D. (G) Co-immunoprecipitation of PDGF receptor with nectin-3 independent of the phosphorylation of PDGF receptor. HEK293 cells transiently expressing PDGF receptor with or without FLAG–nectin-3 were incubated in the presence or absence of 50 µM AG1296, a PDGF receptor inhibitor, for 1 hour. Cell lysates were immunoprecipitated with the anti-FLAG mAb, followed by western blotting as described in D. The results shown in this figure are representative of three independent experiments.

605; #559565) were purchased from Abcam and Pharmingen, respectively. The horseradish peroxidase (HRP)-conjugated and fluorophore-labeled secondary antibodies were obtained from Chemicon and Molecular Probes, respectively.

Cell culture and transfection

NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. Afadin-knockdown NIH3T3 cells were generated as follows: the fragment containing the H1-RNA promoter and short hairpin RNA (shRNA) sequence against afadin was excised from the pBS-H1-afadin vector, which was previously created to transiently knock down afadin as described (Sato et al., 2006), and ligated into the Epstein-Barr-virus-based vector, pEB, which was generated by deletion of the CAG promoter and DsRed gene from pRUBY-M2, which was kindly supplied by Y. Miwa (University of Tsukuba, Tsukuba, Japan), to construct the pEB-H1-afadin vector. NIH3T3 cells stably expressing the shRNA specific for

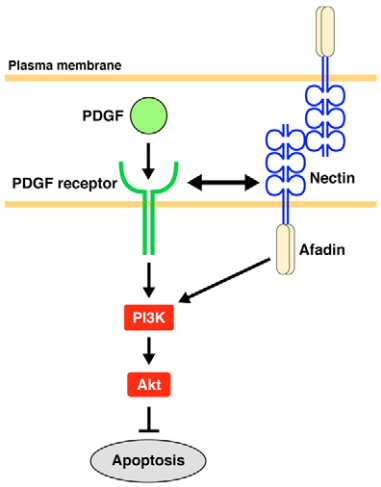

Fig. 7. A schematic model for the crosstalk between the nectin-afadin complex and PDGF-receptor-mediated cell-survival signaling. Details are described in the Discussion.

afadin were generated by the transfection of pEB-H1-afadin into NIH3T3 cells, followed by selection with 500 µg/ml G418 (Nacalai Tesque). Control NIH3T3 cells for afadin shRNA were similarly produced using a scrambled shRNA sequence (5′-CCATCTCAATTCTTGGACG-3′). Control and afadin-knockdown NIH3T3 cells stably expressing GFP or Akt-CA-GFP were produced by additionally transfecting pCAGIpuro-EGFP-N3 or pCAGIpuro-Akt-CA-GFP into control or afadin-knockdown NIH3T3 cells, followed by selection with both 500 µg/ml G418 (Nacalai Tesque) and 10 µg/ml puromycin (Sigma). To knock down nectin-3, a double-stranded 25-nucleotide (nt) RNA duplex (Stealth RNAi; Invitrogen) for nectin-3 (5′-GGACAUUCGCUACUCUUUCAUACUA-3′) was transfected into NIH3T3 cells using the Nucleofection system (Amaxa) following the manufacturer's instructions. As a control for nectin-3 siRNA, a scrambled RNA duplex (5′-GGACUUUCGUCAUUCACUAUCACUA-3′) was also purchased from Invitrogen and transfected into NIH3T3 cells. To knock down nectin-1, a double-stranded 21-nt RNA duplex (Qiagen) for nectin-1 (5′-GGUGGAGGUCAAUAUCACAdTdT-3′) was transfected into NIH3T3 cells. The knockdown of each protein was confirmed by western blotting.

For rescue experiments, expression vectors for RNAi-resistant GFP-afadin (pMSCVpuro-GFP-afadin*), GFP-afadinΔPDZ (pMSCVpuro-GFP-afadinΔPDZ*), nectin-3 (pCAGIPuro-FLAG-nectin-3*) and nectin-3ΔC (pFLAG-CMV1-nectin-3ΔC*) were created by alteration of several nucleotides in the RNAi-target sequence by mutagenesis. AfadinΔPDZ lacks its PDZ domain; this domain mediates the interaction of afadin with nectin. Similarly, nectin-3ΔC lacks the C-terminal four amino acids of nectin-3, which are necessary for binding to afadin. Resistance of each construct to RNAi was confirmed by western blotting. To re-express RNAi-resistant nectin-3 or nectin-3ΔC in nectin-3-knockdown NIH3T3 cells, pCAGIPuro-FLAG-nectin-3* or pFLAG-CMV1-nectin-3ΔC* was transiently transfected into nectin-3-knockdown NIH3T3 cells. To generate stably expressing RNAi-resistant GFP-afadin or GFP-afadinΔPDZ in afadin-knockdown NIH3T3 cells, we performed retrovirus-mediated introduction of each protein into afadin-knockdown NIH3T3 cells using a previously described protocol (Kakunaga et al., 2004). Briefly, when pMSCVpuro-GFP-afadin* or pMSCVpuro-GFP-afadinΔPDZ* was co-transfected with a packing vector pCL-Eco (Imgenex) into HEK293 cells, *GFP-afadin** or *GFP-afadinΔPDZ** gene was packaged into infectious retroviral particles. Then, afadin-knockdown NIH3T3 cells were infected with each of the retroviral particles and selected by 10 µg/ml puromycin in addition to 500 µg/ml G418.

TUNEL staining

Control and afadin-knockdown NIH3T3 cells were serum-starved for 40 hours with or without 100 ng/ml Fas ligand (Jo2 mAb, Pharmingen) in the presence or absence of 3 ng/ml PDGF or EGF at 37°C for 24 hours. After fixation with 4% paraformaldehyde solution on ice for 30 minutes, cells were permeabilized with 0.2% Triton X-100 and analyzed by the TUNEL method (Promega) according to the manufacturer's instructions. The statistical significance of differences in the ratio of TUNEL-positive cells between experimental conditions was analyzed by the Student's *t*-test assuming unequal variance.

EB formation

EBs were generated as described previously with some modifications (Ikeda et al., 1999). Briefly, 129/Sv RW4 wild-type and afadin$^{-/-}$ ES cells were cultured without feeder cells on gelatin-coated dishes for 3 days in high-glucose DMEM supplemented with 20% fetal calf serum (FCS), 0.1 mM 2-mercaptoethanol (Sigma), 1000 U/ml leukemia inhibitory factor (Amrad), 0.1 mM nonessential amino acids (Invitrogen), 3 mM adenosine, 3 mM cytosine, 3 mM guanosine, 3 mM uridine and 1 mM thymidine (Sigma). EB formation was initiated by withdrawal of leukemia inhibitory factor after ES cells had been transferred to bacteriological dishes to grow in suspension culture in DMEM supplemented with 20% FCS. After 9-17 days of suspension culture, EBs were fixed with 4% paraformaldehyde solution on ice for 30 minutes and then suspended in 10% sucrose on ice, followed by replacement with 25% sucrose. Prepared EBs were frozen in OCT compound (Sakura Finetechnical) and sectioned at 10-µm on a cryostat. Sectioned samples were mounted on glass slides, air-dried, washed with PBS and permeabilized with 0.2% Triton X-100 at room temperature for 5 minutes. These samples were analyzed by TUNEL staining as described above. For immunostaining of cleaved caspase-3, the samples were blocked with 1% bovine serum albumin (BSA) and then immunostained with the anti-cleaved-caspase-3 mAb followed by FITC-conjugated secondary antibody.

Western blotting and immunoprecipitation

To examine the phosphorylation levels of various proteins in each group of NIH3T3 cells after PDGF stimulation, cells were serum-starved for 16 hours and treated with 3 ng/ml PDGF at 25°C for the indicated periods of time. After being washed with ice-cold PBS, cells were harvested using pre-warmed Laemmli buffer (Laemmli, 1970) containing 1 mM Na_3VO_4, 10 mM NaF and a phosphatase inhibitor cocktail (Sigma), boiled for 5 minutes and sonicated three times for 10 seconds with 20-second cooling periods. The protein concentrations of the samples were determined using an RC DC protein assay kit (Bio-Rad) with BSA as a reference protein. The samples were separated by SDS-PAGE and this was followed by western blotting with the indicated phospho-specific antibodies. Densitometric analysis was performed using ImageJ software (National Institutes of Health) and paired Student *t*-tests were performed for statistical analysis. For the immunoprecipitation assay, NIH3T3 cells or HEK293 cells expressing various combinations of the indicated molecules were lysed with Buffer A (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% NP-40, 1 mM APMSF, 3 µg/ml leupeptin and 5 µg/ml aprotinin). To examine the association of PDGF receptor with nectin-3 endogenously in NIH3T3 cells, we used a chemical cross-linker, 3,3′-dithio-bis (sulfosuccinimidylpropionate) (DTSSP) (Pierce) and prepared cell lysates as described (Minami et al., 2007). The cell lysates were centrifuged at 100,000 ***g*** for 15 minutes and the supernatant was then incubated with the anti-FLAG mAb or anti-PI3K (p85) pAb at 4°C for 2 hours, followed by incubation with protein G- or protein A-Sepharose beads at 4°C for 2 hours. After the beads had been extensively washed with Buffer A, bound proteins were eluted from the beads by boiling in SDS sample buffer for 5 minutes and were subjected to SDS-PAGE, followed by western blotting with the indicated antibodies.

Assay for PI3K activity

PI3K activity was assayed as previously described with some modifications (Ijuin and Takenawa, 2003). Control, nectin-3-knockdown and afadin-knockdown NIH3T3 cells were serum-starved for 16 hours and stimulated with or without 3 ng/ml PDGF at 25°C for 2 minutes. The cells were washed once with cold PBS and lysed with Buffer B (20 mM Tris-HCl at pH 7.5, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2 M NaCl, 10% glycerol, 1% NP-40, 2 mM PMSF and 100 µM Na_3VO_4). The cell lysates were centrifuged at 100,000 ***g*** for 15 minutes and supernatants were incubated with the anti-PI3K (p85) pAb at 4°C for 4 hours. The immune complex was collected with Protein A-Sepharose beads and re-suspended in 100 µl of kinase buffer (100 mM Tris-HCl at pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA and 0.1 mM EGTA) and incubated with [γ-^{32}P]ATP (3,000 Ci/mmol) and phosphatidylinositol as substrate at 37°C for 10 minutes. The reaction was terminated by the addition of 100 µl of 1 N HCl. The lipids were extracted with chloroform-methanol (2:1, vol:vol) and applied to a thin-layer chromatography plate (Merck). The plate was developed in chloroform-methanol-H_2O-ammonium (60:47:11.6:2, vol:vol:vol:vol), dried and visualized by autoradiography with an X-ray film. The radioactivity of each spot was quantified using a scintillation counter.

Immunofluorescence microscopy and bead-cell contact assay

Immunofluorescence microscopy was performed as described previously (Sato et al., 2006). Briefly, NIH3T3 cells were fixed with 4% formaldehyde at 25°C for 15 minutes or with a mixture of 50% acetone and 50% methanol on ice for 1 minute. After being blocked with 1% BSA, cells were immunostained with the first antibody for 1 hour, followed by incubation with fluorophore-labeled secondary antibody for 30 minutes. The samples were analyzed using an LSM510 META confocal microscope (Carl Zeiss).

Bead-cell contact was assayed as previously described (Honda et al., 2003b). Briefly, NIH3T3 cells were seeded onto coverslips, cultured for 3 hours and then incubated with latex-sulfate microbeads coated with Nef-1 for 1 hour. After incubation, cells were fixed and immunostained with the indicated antibodies, followed by observation using the above confocal microscope.

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