Integrin-linked kinase is involved in lactoferrin-induced anchorage-independent cell growth and survival in PC12 cells

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Abstract

Aims: Bovine lactoferrin (bLf) causes anchorage-independent cell growth in PC12 cells. The present study investigated the mechanisms involved in bLf-induced anchorage-independent cell growth and survival in PC12 cells.

Main methods: The number of adherent cells and suspended cells was estimated separately by using a methyl thiazol tetrazolium (MTT) assay, and the sum of both optical density (O.D.) (570 nm) values was used as a measure of the total number of cells.

Key findings: Integrin-linked kinase (ILK) plays an important role in integrin and growth factor signaling pathways. Stable transfection of PC12 cells with a dominant negative kinase-deficient mutant of ILK (DN-ILK) inhibited bLf-induced anchorage-independent cell growth. The ILK activity in the parental cells was transiently activated after addition of bLf, whereas bLf-induced activation of ILK was blocked in DN-ILK-transfected cells. bLf also activated p38 mitogen-activated protein kinase (MAPK); however, the p38 MAPK activation was inhibited by stable DN-ILK transfection. Moreover, cell viability in the suspended cells by bLf strongly decreased after treatment with SB203580, an inhibitor of p38 MAPK.

Significance: These results suggest that ILK is involved in bLf-induced anchorage-independent cell growth and viability via activation of p38 MAPK.

Key words: Bovine lactoferrin; PC12 cell; Integrin-linked kinase; Integrin; p38 MAPK; Cell suspension; Cell adhesion; Cell growth; Cell viability; Dominant-negative mutant; Anchorage-independent growth

Introduction

Lactoferrin is an iron-binding glycoprotein present in various exocrine fluids such as milk, tears, saliva, and bile (Vorland, 1999). Lactoferrin is also a major constituent of the secondary granules of neutrophilic leukocytes, and it is released from these granules during acute inflammation (Caccavo et al., 1999). Cornish et al. (2004) demonstrated that lactoferrin potently stimulates the proliferation and differentiation of osteoblasts but inhibits osteoclastogenesis, resulting in the promotion of bone growth. Moreover, we previously demonstrated that bovine lactoferrin (bLf) binds to the membrane of PC12 cells via membrane-associated proteoglycans, leading to anchorage-independent growth (Ishii et al., 2007). Thus, lactoferrin is a growth factor, but its signaling mechanisms remain unknown.

Integrin-linked kinase (ILK) is a cytoplasmic serine/threonine protein kinase that mediates integrin- and growth factor-stimulated signal transduction (Dedhar et al., 1999; Huang and Wu, 1999; Ishii, 2005). ILK is expressed in PC12 cells and activated by nerve growth factor (NGF) (Mills et al., 2003). Moreover, inhibition of endogenous ILK, either by a pharmacological inhibitor or by overexpression of a dominant-negative mutant of ILK (DN-ILK), blocks the NGF-mediated neurite outgrowth in PC12 cells (Mills et al., 2003) and the integrin-dependent neurite outgrowth in N1E-115 neuroblastoma cells (Ishii et al., 2001). Thus, ILK functions as an important effector in integrin and growth factor–mediated neuronal differentiation and growth. In the present study, we examine the functional role of ILK in bLf-induced anchorage-independent growth and viability in suspended cells. We also characterize the signaling pathways used in these processes.

Materials and methods

Reagents

PC12 cells were obtained from the Riken Cell Bank (Tsukuba, Japan). A Cell Proliferation Kit 1 (MTT) and a biotin-labeling kit were obtained from Roche Diagnostics (Penzberg, Germany). The following reagents were purchased for this study: Dulbecco's Modified Eagle Medium (DMEM) (GIBCO BRL; Rockville, MD), Zeocin (Invitrogen; Tokyo, Japan), BLf (Wako Pure Chemical Co.; Osaka, Japan), fetal bovine serum (Hyclone; Logan, UT), and SB203580 (Calbiochem; San Diego, CA). Anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-MEK, anti-MEK, anti-phospho-ERK, and anti-ERK were obtained from Cell Signaling Technology (Beverly, MA). Anti-ILK antibody (UB 06-592) was obtained from Upstate Biotechnology (Lake Placid, NY). All other chemicals were analytical grade and were obtained from Sigma-Aldrich (Tokyo, Japan) or Wako Pure Chemical Co.

Construction and transfection of cDNA vectors and cell culture

DN-ILK was generated by site-directed mutagenesis (Glu to Lys) of amino acid residue 359, which lies within the kinase domain of wild-type ILK (GenBankTM accession number AF256520), by using a PCR-based method, as described previously (Ishii et al., 2001). DN-ILK cDNA was ligated into the polylinkers in the mammalian expression vector pTracerTM-CMV2 (V885-01; Invitrogen). The DN-ILK cDNA was transfected into PC12 cells by calcium phosphate precipitation, and 48 individual zeocin-resistant cell lines were isolated during the next 4 to 5 weeks, as described previously (Ishii et al., 2001; Ishii et al., 2003). Of these 48 cell lines, 3 were selected based on the presence of green fluorescent protein fluorescence and by confirmation of gene transcription by using reverse transcription-PCR. The cloned cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were treated with bLf by direct addition to the culture medium.

Cell proliferation and viability assay

Cells were seeded on 96-well plates and 35-mm culture dishes at a density of 7×10^3 cells/well and 7×10^4 cells/dish, respectively, and cultivated for 24 h. Cells were then treated with varying concentrations of bLF for 16 h by direct addition to the culture medium. Cell suspension occurs immediately after the direct addition of bLf to the culture medium. The number of suspended cells increased time-dependently but was retained constantly over 16 h after the addition of bLf. The culture medium containing suspended cells was transferred to new wells or plates, and new culture medium was added to the original wells or dishes containing the adherent cells. The number of adherent cells and suspended cells was estimated separately by using a methyl thiazol tetrazolium (MTT) assay (Cell Proliferation Kit 1; Roche Diagnostics), and the sum of both optical density (O.D.) (570 nm) values was used as a measure of the total number of cells, as described previously (Ishii et al., 2007).

ILK assay

ILK activity was measured by assessing the phosphorylation of inactive Akt, as described by Kagami et al. (2006). Cells were lysed in ice-cold lysis buffer (Cell Signaling Technology, Inc.) in the presence of protease inhibitors (Roche Diagnostics, Indianapolis, IN), and the cell lysates were incubated with an anti-ILK antibody. Immune complexes were collected with protein G-Sepharose and incubated with kinase buffer (Upstate Biotechnology; Lake Placid, NY) supplemented with 200 mM ATP and 250 ng inactive recombinant Akt/PKBa (Upstate Biotechnology). Phosphorylated Akt was analyzed by Western blotting with phospho-Akt (Ser473) antibody (Cell Signaling Technology, Inc.). Endogenous ILK protein levels in cell lysates were analyzed by Western blotting with anti-ILK antibody. The blots were probed with appropriate primary antibodies, followed by goat anti-rabbit IgG

conjugated to horseradish peroxidase (Vectastain ABC kit; Burlingame, CA), and the final protein-IgG complexes were detected by using chemiluminescence reagents (GE Healthcare; Buckinghamshire, UK).

Statistical methods

To evaluate the effect of bLf on cell growth and adhesion in parental and DN-ILK-transfected cells, data were analyzed by one-way analysis of variance (ANOVA) after a Levene test. Either the Tukey-Kramer or Dunnett test was used as a post hoc test. To evaluate the effect of SB203580 on cell growth and adhesion in the presence or absence of bLf, data were analyzed by either the Student's *t* test or Wilcoxon signed-ranks test after a Levene test. *P* values less than 0.05 were considered statistically significant.

Results

bLf inhibits cell adhesion and leads to anchorage-independent growth

PC12 cells adhere well to plastic tissue culture plates and show anchorage-dependent cell growth. We previously reported that bLf binds to the membrane of PC12 cells via membrane-associated proteoglycans and prevents cell adhesion to plastic tissue culture plates (Ishii et al., 2007). To confirm our previous observation, PC12 cells were treated with varying concentrations (1 ~ 150 μM) of bLf for 16 h. As shown in Fig. 1, bLf dose-dependently inhibited cell adhesion and caused the cells to be released into suspension. Total cell number, however, was not affected in the presence of bLf (1 ~ 150 μM). Thus, we confirmed that bLf inhibits cell adhesion and leads to anchorage-independent cell growth in PC12 cells.

The bLf-induced anchorage-independent growth of parental cells requires the transient activation of ILK

ILK is a cytoplasmic serine/threonine protein kinase that mediates integrin- and growth factor–stimulated signal transduction (Dedhar et al., 1999; Huang and Wu, 1999; Ishii, 2005). ILK is expressed in PC12 cells and functions as an important effector in integrin- and growth factor–mediated neuronal differentiation and growth (Mills et al., 2003). Therefore, we examined whether bLf activates endogenous ILK. As shown in Fig. 2A, the ILK activity in the parental cells was transiently activated after addition of bLf, whereas the bLf activation of ILK was blocked in DN-ILK–transfected cells. Maximal stimulation of ILK activity in the parental cells occurred 5-10 min after addition of bLf and then rapidly diminished. bLf dose-dependently activated ILK activity when the parental cells were treated with bLf in the concentration range of 30 ~ 150 μM for 10 min. Maximum activation of ILK occurred in the presence of bLf of 100 μM and over (date not shown). In contrast to parental cells, bLf-induced detachment was significantly inhibited in DN-ILK-transfected cells (Fig. 2B). Moreover, treatment of the DN-ILK–transfected cells with higher concentrations of bLf (> 100 μM) resulted in a slight but significant decrease in the total cell number (Fig. 2B). These results suggest that endogenous ILK is involved in bLf-induced inhibition of cell adhesion and anchorage-independent cell growth.

P38 MAPK but not MEK/ERK is activated after treatment of parental cells with bLf, and endogenous ILK is involved in this activation

Both the p38 MAPK and the MEK/ERK pathways are critical for growth factor–mediated and integrin-mediated growth and neuronal differentiation (Ishii et al., 2001; Cowley et al., 1994; Morooka and Nishida, 1998). Therefore, we examined the possible

involvement of p38 MAPK and MEK/ERK in the signaling pathway of bLf-induced anchorage-independent cell growth. As shown in Fig. 3A, p38 MAPK in the parental cells was transiently activated after addition of bLf. This activation peaked within 60 min after the addition of bLf, and then it decreased to the basal level within 120 min. Thus, p38 MAPK activation occurred after ILK activation in the time course following the addition of bLf (Fig. 2A and Fig. 3A). In contrast, p38 MAPK was not activated in the DN-ILK–transfected cells after the addition of bLf (Fig. 3A). MEK and ERK were activated within 60 min and 5 min, respectively, after the addition of bLf, and then the activations decreased to their basal level (Fig. 3B and C). In contrast to p38 MAPK, the bLf-induced activation of MEK and ERK was not blocked in DN-ILK–transfected cells (Fig. 3). Moreover, the basal activities of both MEK and ERK were observed in the presence or absence of bLf, and these basal activities in DN-ILK–transfected cells were much higher than the activities in parental cells but tended to be a little lower after the addition of bLf (Fig. 3). Thus, although p38 MAPK, MEK and ERK in the parental cells are transiently activated by bLf, only bLf-induced activation of p38 MAPK is dependent on the endogenous ILK activity. These results suggest that p38 MAPK is activated by bLf and that endogenous ILK is somehow involved in the bLf-induced activation of p38 MAPK.

Activation of p38 MAPK is critical for bLf-induced anchorage-independent cell growth and viability in suspended cell

To evaluate the specific role of the p38 MAPK signaling pathway in bLf-induced anchorage-independent growth and viability, PC12 cells were treated with SB203580, a specific inhibitor of p38 MAPK. The cells were cultivated for 16 h in medium containing 5 μM SB203580 in the presence or absence of 100 μM bLf. Treatment of DN-ILK–transfected cells

with SB203580 stimulated cell growth in either the presence or absence of bLf (Fig. 4A, C). In contrast to DN-ILK-transfected cells, treatment of parental cells with SB203580 strongly decreased the total cell number in the presence of bLf but not in the absence of bLf, indicating that SB203580 leads to decreased cell viability only in the presence of bLf (Fig. 4A, B). Moreover, the decreased number of total cells mostly corresponded to the decreased number of suspended cells, suggesting that p38 MAPK is involved in cell viability in the bLf-induced cell suspension (Fig. 4B right). In the absence of bLf, SB203580 did not affect the total cell number, but there was a slight increase in the proportion of cells that were suspended (Fig. 4B). These results suggest that the signaling pathway of p38 MAPK, which can be activated via the bLf-induced activation of ILK, is required for cell survival in suspended cells by bLf.

Discussion

PC12 cells adhere well to plastic culture plates and show anchorage-dependent cell growth. When bLf was added to the culture medium, the cells immediately detached and began to form clusters in suspension (Ishii et al., 2007). We have previously demonstrated that bLf stimulates anchorage-independent growth via membrane-associated chondroitin sulfate and heparin sulfate proteoglycans in PC12 cells (Ishii et al., 2007). Furthermore, we also found that integrin-dependent cell adhesion on a laminin matrix prevents bLf-induced detachment of PC12 cells (Ishii et al., 2007). These findings imply that bLf might affect some integrin-linked intracellular signaling pathway after its binding to the membrane-associated proteoglycans and result in bLf-induced inhibition of cell adhesion and the promotion of growth in suspension.

Integrin-linked kinase (ILK) is a cytoplasmic serine/threonine protein kinase that mediates integrin- and growth factor–stimulated signal transduction (Dedhar et al., 1999; Huang and Wu, 1999; Ishii, 2005). ILK is expressed in PC12 cells and is activated by nerve

growth factor (NGF) (Mills et al., 2003). To investigate the role of endogenous ILK in the bLf-induced anchorage-independent cell growth and viability of PC12 cells, we cloned cell lines that highly express a kinase-deficient mutant of ILK (DN-ILK), which behaves as a dominant negative (Mills et al. 2003; Cornish et al., 2004; Ishii et al., 2007). We found that ILK activity in the parental cells was transiently activated after the addition of bLf, whereas the bLf activation of ILK was blocked in DN-ILK–transfected cells. Moreover, bLf-induced detachment of cells was significantly inhibited in DN-ILK–transfected cells. These results suggest that bLf activates the ILK-signaling pathway that permits anchorage-independent growth. Although bLf activated ILK, its activation mechanism remains unknown. bLf binds to the membrane of PC12 cells via membrane-associated proteoglycans (Ishii et al., 2007); thus, there may be cross-talk of signals between the membrane-associated proteoglycans and ILK.

PC12 cells have been used in studies of neuronal differentiation induced by neurotrophic factors such as NGF (Cowley et al., 1994; Morooka and Nishida, 1998). These studies suggest that growth factor–induced activation of MAPK pathways is critical not only for neuronal differentiation but also for cell viability. On the other hand, integrin-dependent adhesion induces MAPK activation (Zhu and Assoian, 1995) and cooperates with growth factors to efficiently activate the MAPK pathway (Miyamoto et al., 1996). A recent study of neuronal differentiation in N1E-115 neuroblastoma cells demonstrated that ILK activation induces transient activation of p38 MAPK (Ishii et al., 2001). Cross-talk between ILK and MAPK has been reported in studies on anchoring-independent cell cycle progression (Wang et al., 1998). Therefore, we examined the possible involvement of MAPK in the signaling pathway of bLf-induced anchorage-independent cell growth. P38 MAPK in the parental cells was transiently activated after addition of bLf, and this activation was blocked by the overexpression of DN-ILK. MEK and ERK in the parental cells were also transiently activated

after the addition of bLf; however, the bLf-induced activation of MEK and ERK was not blocked in DN-ILK–transfected cells (Fig. 3). These results suggest that ILK activation is necessary for bLf-induced activation of p38 MAPK but not MEK or ERK.

We next examined the role of the p38 MAPK signaling pathway in bLf-induced anchorage-independent growth and viability in PC12 cells. SB203580, a specific inhibitor of p38 MAPK, stimulated cell growth in either the presence or absence of bLf in DN-ILK–transfected cells. Neither ILK nor p38 MAPK activation was detected in the DN-ILK–transfected cells after treatment with bLf; the reason for this observation is not known. In contrast to DN-ILK–transfected cells, treatment of parental cells with SB203580 strongly decreased the total cell number in the presence of bLf but not in the absence of bLf. Moreover, the decreased number of total cells mostly corresponds to the decreased number of suspended cells. Inhibition of p38 MAPK, therefore, might lead to low cell viability in the bLf-induced cell suspension. These results suggest that the signaling pathway of p38 MAPK is required for cell survival in the suspended cells by bLf.

Basal activity of p38 MAPK was observed in parental cells even in the absence of bLf. When the parental cells were treated with SB203580 in the absence of bLf, the number of suspended cells increased but that of adherent cells decreased without affecting total cell number (Fig.4B). These results suggest that the basal p38 MAPK activity is partly involved in inhibition of anchorage-independent growth of parental cells. Moreover, because basal activity of p38 MAPK was not observed in DN-ILK-transfected cells, it seems that ILK is necessary for maintenance of the basal p38 MAPK activity. On the other hand, when DN-ILK-transfected cells, in which basal activity of p38 MAPK was not observed, were treated with SB203580, SB203580 stimulated cell growth of the DN-ILK-transfected cells notwithstanding the presence or absence of bLf (Fig.4C). These results imply that SB203580 might have a

stimulatory effect of cell growth other than an inhibitory effect of p38 MAPK. However, detailed mechanisms of the stimulatory effect of SB203580 on cell growth observed in DN-ILK-transfected cells remain unknown.

It has been well known that a transient ILK activation leads to activation of various growth-signaling proteins such as Akt and Nck-2 other than p38 MAPK (Ishii, 2005). These proteins might be involved in bLf-induced anchorage-independent cell growth. A further study needs to unravel the molecular relationship between ILK and other growth-signaling molecules than p38 MAPK in bLf-induced anchorage-independent cell growth in PC12 cells.

Conclusion

In conclusion, we found that ILK in PC12 cells is involved in bLf-induced anchorage-independent cell growth and viability and also that the activation of p38 MAPK is critical to these effects of ILK, especially cell survival in suspended cells by bLf. Further studies are required to elucidate the molecular mechanisms of bLf-induced activation of ILK.

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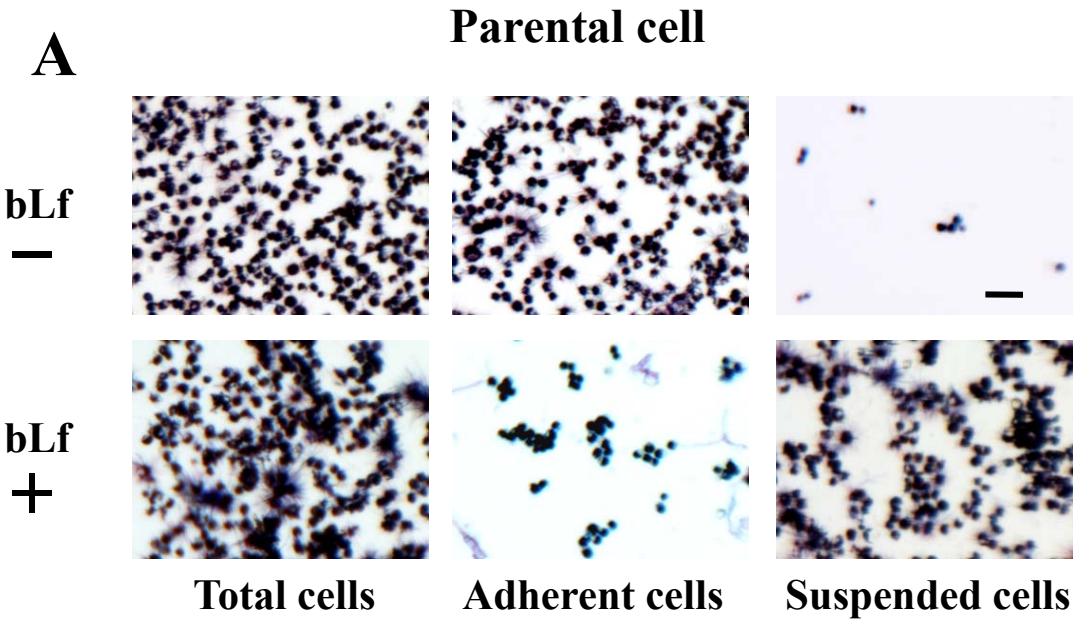
Figure legends

Fig. 1. Effects of bLF on cell growth and adhesion of parental cells on plastic tissue culture plates. Cells were treated with varying concentrations of bLf for 16 h by direct addition to the culture medium. (A) Cells were cultured in the presence or absence of 100 μM bLf for 16 h. Photomicrographs were taken 3 h after the addition of MTT reagent. The scale bar indicates 50 μm. (B) The numbers of adherent and suspended cells were estimated individually by using an MTT assay, and the sum of both O.D. values was used as an indication of the total number of cells. Results represent means ± SDs of O.D. values from 6 independent cultures. $^{*}P<0.05$ vs. untreated suspended cells. $^{+}P<0.05$ vs. untreated adherent cells.

Fig. 2. Effects of bLf on ILK activity in parental cells and DN-ILK–transfected cells and on cell growth and adhesion of DN-ILK–transfected cells on plastic tissue culture plates. (A) Cells grown on plastic tissue culture plates were cultured for the indicated time periods after addition of 100 μM bLf. The cells were lysed, and ILK was immunoprecipitated from cell extracts. ILK activity was determined by assessing the phosphorylation of inactive Akt, as described by Kagami et al. (2006). Endogenous ILK protein levels were analyzed by Western blotting of cell lysates with an anti-ILK antibody. (B) Cells were treated with varying concentrations of bLf for 16 h by direct addition to the culture medium. The number of adherent and suspended cells was estimated individually by using an MTT assay, and the sum of both O.D. values was used as an indication of the total number of cells. Results represent the means ± SDs of O.D. values from 6 independent cultures. $^{*}P<0.05$ vs. untreated suspended cells; $^{+}P<0.05$ vs. untreated adherent cells; and $^{\#}P<0.05$ vs. untreated total cells.

Fig. 3. Effects of bLf on p38 MAPK (A), MEK (B), and ERK (C) in parental cells and DN-ILK–transfected cells. Cells grown on normal plastic tissue culture plates were cultured for the indicated time periods after addition of 100 μM bLf. The cells were lysed, and the lysates were subjected to immunoblotting analysis. The active forms of p38 MAPK (A top), MEK (B top), and ERK (C top) were detected with antibodies against the phosphorylated forms of these proteins. Antibodies against p38 MAPK (A bottom), MEK (B bottom), and ERK (C bottom) were used to detect the total levels (both phosphorylated and unphosphorylated) of these proteins. The blots were probed with appropriate primary antibodies, followed by goat anti-rabbit IgG conjugated to horseradish peroxidase. The final protein-IgG complexes were detected by using chemiluminescence reagents.

Fig. 4. Effect of SB203580 on anchorage-independent cell growth and viability. (A) Both parental and DN-ILK–transfected cells were cultivated for 16 h in medium containing 100 μM bLf in the presence or absence of 5 μM SB203580. Photomicrographs were taken 3 h after the addition of the MTT reagent, which stains only living cells. The scale bar indicates 100 μm. (B) and (C) The cells were cultivated for 16 h in medium containing 5 μM SB203580 in the presence or absence of 100 μM bLf. The number of adherent and suspended cells was estimated individually by using an MTT assay, and the sum of both O.D. values was used as an indication of the total number of cells. Results represent the means ± SDs of O.D. values from 6 independent cultures. $^{*}P<0.05$ vs. SB203580-untreated suspended cells; $^{+}P<0.05$ vs. SB203580-untreated adherent cells; and $^{\#}P<0.05$ vs. SB203580-untreated total cells.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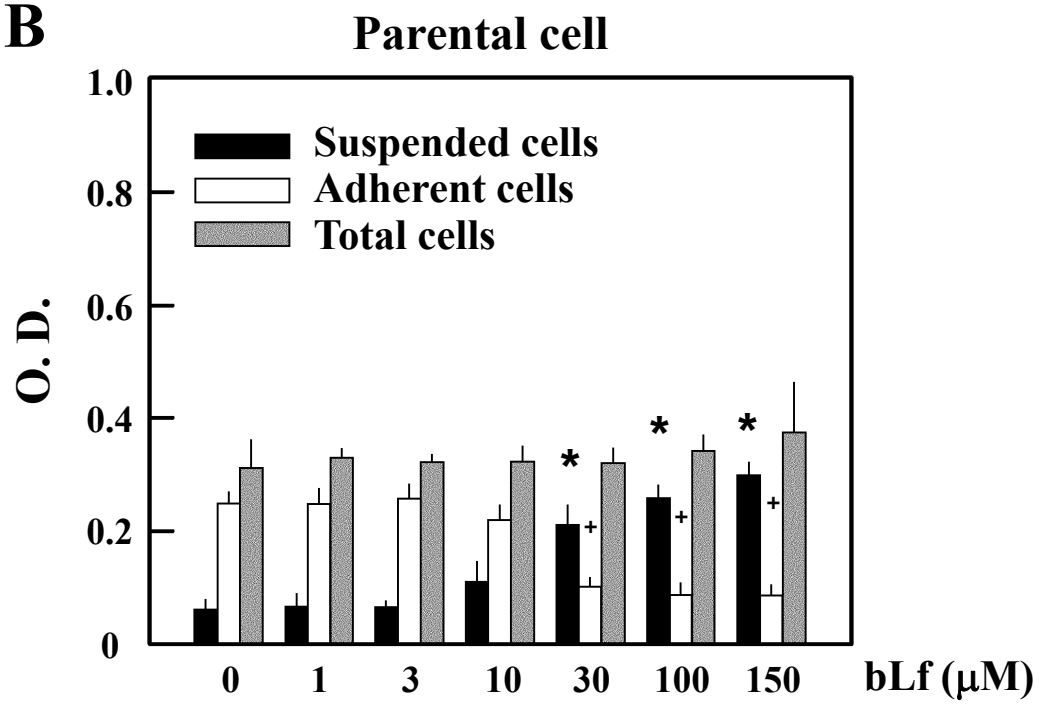
bLf
−
bLf
+
Total cells
Adherent cells
Suspended cells


B
Parental cell
Suspended cells
Adherent cells
Total cells
O. D.
1.0
0.8
0.6
0.4
0.2
0
0
1
3
10
30
100
150
bLf (μM)
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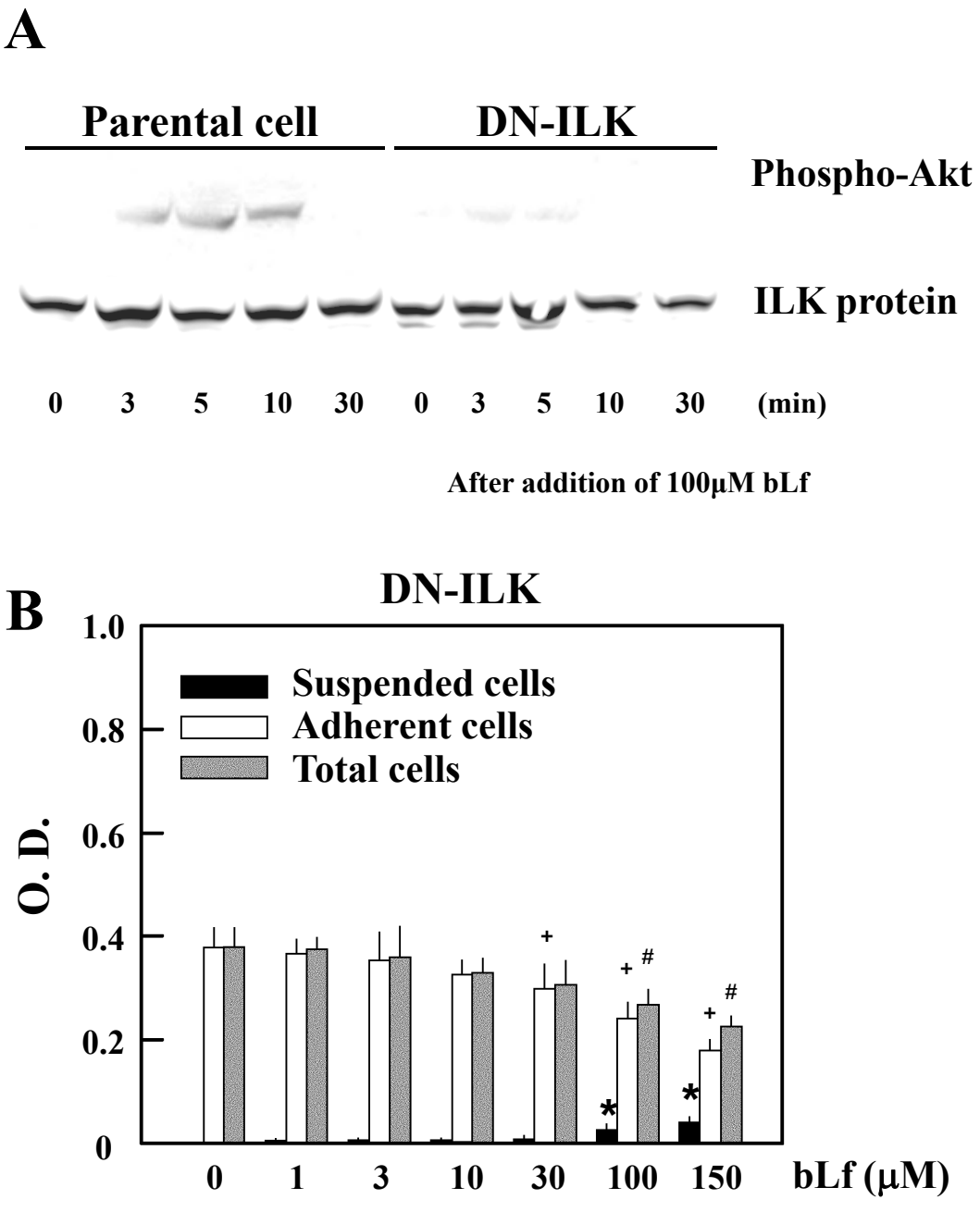
*
*
+
+
+

Fig. 1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Parental cell
DN-ILK
Phospho-Akt
ILK protein
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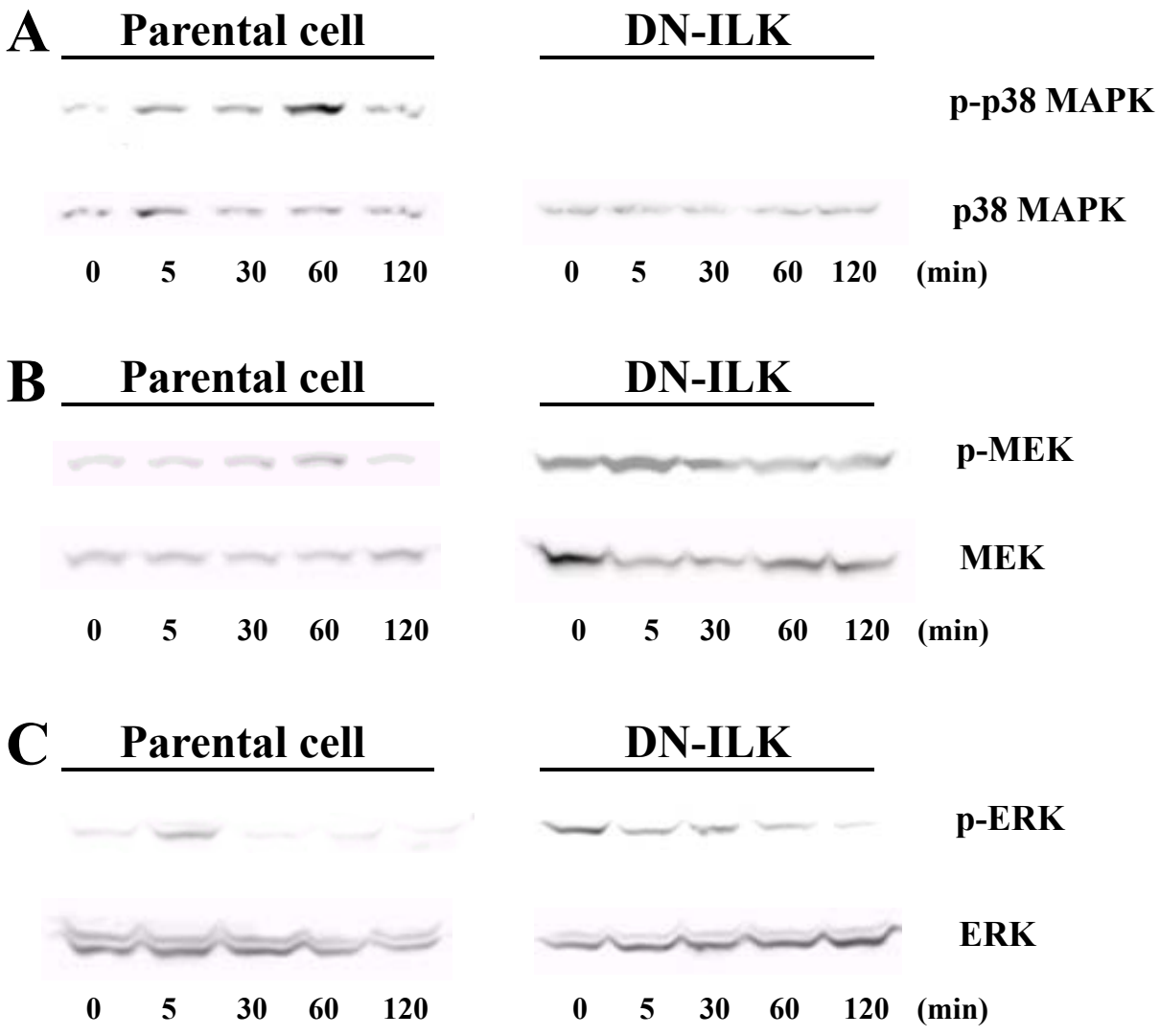
After addition of 100μM bLf
B
DN-ILK
Suspended cells
Adherent cells
Total cells
O. D.
1.0
0.8
0.6
0.4
0.2
0
0 1 3 10 30 100 150 bLf (μM)

Fig. 2


A
Parental cell
DN-ILK
p-p38 MAPK
p38 MAPK
0 5 30 60 120
0 5 30 60 120 (min)
B
Parental cell
DN-ILK
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MEK
0 5 30 60 120
0 5 30 60 120 (min)
C
Parental cell
DN-ILK
p-ERK
ERK
0 5 30 60 120
0 5 30 60 120 (min)

Fig. 3

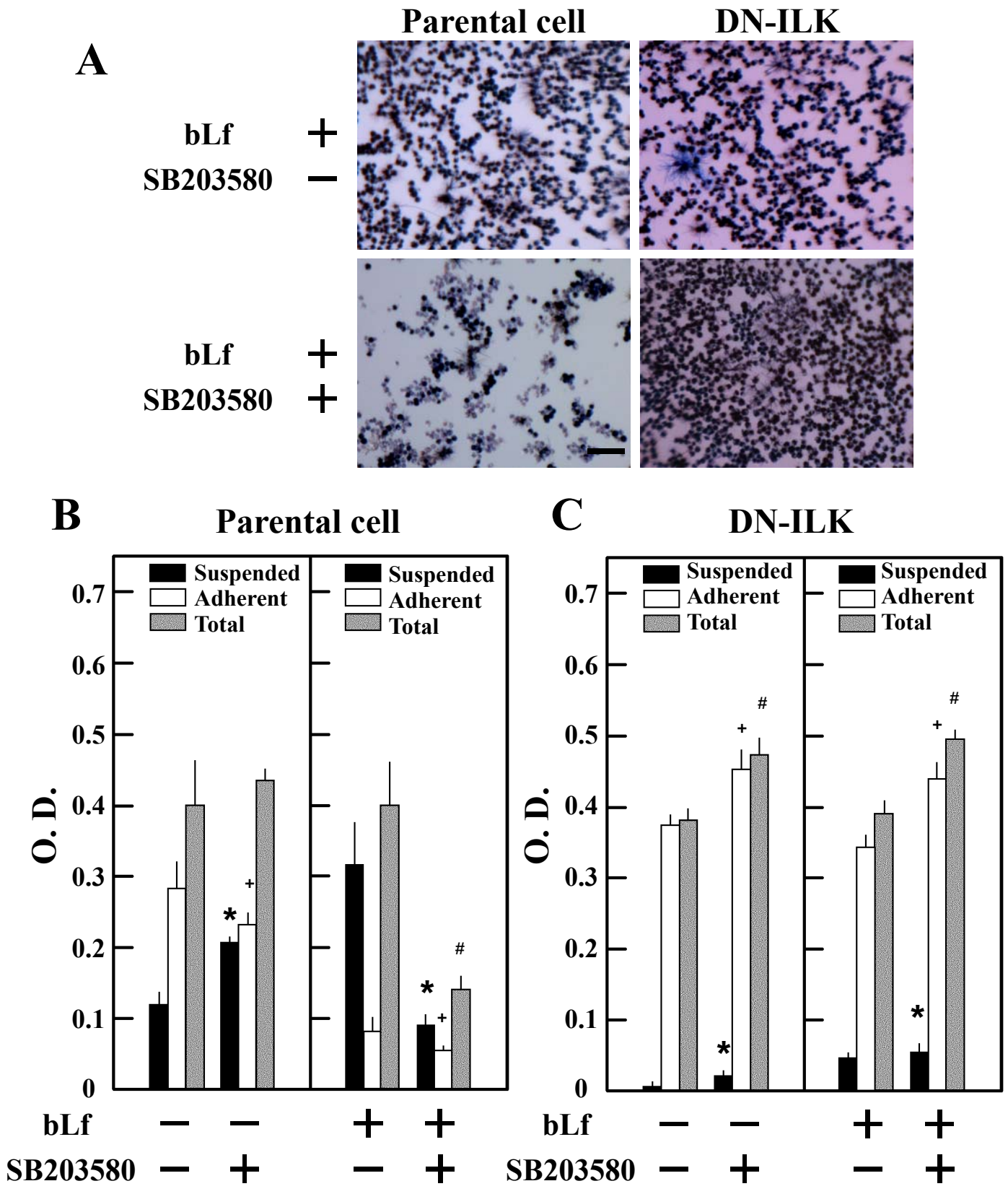
A
Parental cell
DN-ILK
bLf +
SB203580 −
bLf +
SB203580 +
B
Parental cell
C
DN-ILK
Suspended
Adherent
Total
O. D.
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
bLf
SB203580

Fig. 4