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Title

An improved method for western blotting when extracting proteins from mammalian cells cultured on a collagen gel under serum-free conditions

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Abstract

Western blotting is a widely used method for detection and quantification of specific proteins extracted from mammalian cells. In the conventional method of protein extraction, we found that collagen-containing gels interfered with detection of the p65 protein (one of the subunits in the NF-κB family of proteins) in human lung adenocarcinoma A549 cells cultured on a collagen gel containing serum. In contrast, the collagen gels did not affect detection of the GAPDH protein. Then, we established an improved method for preparation of protein extracts (using trichloroacetic acid fixation and collagenase treatment) from the cells cultured on the collagen gel. Using the improved method, we were able to detect p65 proteins without a loss in A549 cells cultured on a collagen gel under serum-free conditions, but we could not if serum was present in cell culture. Thus, using western blotting and serum-free culture conditions, we succeeded in comparing the p65 expression between the cells grown in a plastic dish and cells grown on a collagen gel.

Keywords

western blotting; collagen gel; serum; trichloroacetic acid; collagenase; p65

Introduction

Western blotting (WB) is one of the well-established methods for detecting and quantifying specific proteins (Laemmli 1970; Towbin et al. 1979). A recent study reported that substrate stiffness affects cell behavior. For example, the rigidity of a collagen matrix regulates collective motility and the expression of adhesion proteins (Wang et al. 2003; Haga et al. 2005). Furthermore, stiffer substrates may enhance cancer progression (Paszek et al. 2005, Levental et al. 2009). Accordingly, quantitative analysis of specific proteins in the cells cultured on stiff and soft substrates is gaining wider interest. In particular, the proteins that regulate intracellular biochemical signaling affected by substrate stiffness have been analyzed. NF-κB is one of the regulators of such signaling and plays important roles in inflammatory reactions and cancer progression (Karin et al. 2002; Li and Verma 2002; Ishihara et al. 2013). We focused on the p65 protein, one of the NF-κB subunits.

In this study, we found that collagen-containing gels hamper detection of the p65 protein in the cells cultured on a collagen gel containing serum. Then, we established an improved method for western blotting using trichloroacetic acid (TCA) fixation and collagenase treatment to extract proteins from mammalian cells cultured on a collagen gel under serum-free conditions. TCA is used as a fixative of cells or tissues for various experiments, such as western blotting and histochemical analysis (Cafruny 1957; Musial and Eissa 2001). Although the mechanism has not been identified yet, TCA fixation is thought to be caused by reactions between the negatively charged chloroacetate groups of TCA and positively charged amino groups of the proteins. Collagenases are enzymes that hydrolyze the triple helical region of collagens (Bond and Van Wart 1984). Thus, by means of the collagenase treatment, we were able to dissolve the collagen gel and remove it from cell lysates. In previous studies, researchers often lysed the cells without trichloroacetic acid and collagenase treatment for western blotting (Wang et al.

2003; Engler et al. 2006; Tsutsumi et al. 2009; Wu et al. 2009). We refer to this approach as “the conventional method” in this study. Compared to the conventional method, our improved method allowed for detection of the band of p65 proteins without losses.

Materials and methods

Cell culture and material preparation

The A549 human lung adenocarcinoma cell line (American Type Culture Collection, Manassas, VA), HT1080 human fibrosarcoma cell line (Riken Cell Bank, Tsukuba, Japan), and NIH-3T3 mouse fibroblast cell line (Riken Cell Bank) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma, St Louis, MO) containing 10% fetal bovine serum (BIST TECH, Equitech Bio Inc., Kerrville, TX or Biowest, Nuaillé, France) and 1% antibiotic/antimycotic solution (Sigma). Mouse primary fibroblasts were isolated from the abdominal subcutaneous tissue of a male Slc:ICR retired mouse (Japan SLC, Inc., Hamamatsu, Japan). The animal experiment was strictly compliant with the animal care guidelines of Hokkaido University. The cells were incubated at 37 °C in a humidified incubator with 5% CO_2. Each cell type was passaged and used for experiments unless the cells showed aberrant morphology. For western blotting, 10^5 cells were seeded on a 35 mm plastic dish (>1 GPa) coated with type I collagen (Cell matrix I-C; Nitta Gelatin, Osaka, Japan) or on a 500 μL of type I collagen gel (1.6 mg/mL, approximately 0.6 kPa; Cell matrix I-P, Nitta Gelatin) in a 35 mm plastic dish. The cells were cultured for 24 h for western blotting. For serum-free culture, we seeded 10^5 cells in a culture dish filled with serum-free DMEM and cultured them for 24 h. To add a collagen gel to a cell lysate, we prepared 500 μL of type I collagen gel (1.6 mg/mL, approximately 0.6 kPa; Cell matrix I-P, Nitta Gelatin) in a 35-mm plastic dish. The gel was covered with serum-containing or serum-free DMEM and incubated at 37 °C in a humidified incubator with 5% CO_2 for 24 h.

Antibodies

For western blotting, we used the following antibodies: an anti-NF-κB p65 antibody (IBL, Fujioka, Japan; cat. # 18667), anti-GAPDH (Ambion, Foster City, CA; cat. # 1103016),

anti-integrin β1 (BD BioScience, San Jose, CA; cat. # 610467), anti-cyclin E1 (Cell Signaling Tech, Danvers, MA; cat. # 4129), HRP-conjugated anti-rabbit IgG (Cell Signaling Tech; cat. # 7074), and an HRP-conjugated anti-mouse IgG antibody (Bio-Rad, Hercules, CA; cat. # 70-6516).

Western blotting

In the conventional method for extraction of proteins from the cells cultured in a plastic dish and on a collagen gel, we removed the medium from the cell sample and lysed the cells with 200 μL of SDS sample buffer (0.25 M Tris-HCl pH 8.8, 5% dithiothreitol, 2.3% SDS, 10% glycerol, 0.01% bromophenol blue). This SDS sample buffer was used for lysing the whole-cell extract. After that, the cell lysate was homogenized, sonicated using an ultrasonic homogenizer (UH-50; SMT Company, Tokyo, Japan), and then boiled at 95 °C for 5 min.

In the improved method for extraction of proteins from the cells cultured in a plastic dish, we removed the media from the cell sample and added 500 μL of 10% trichloroacetic acid (TCA, Sigma) in phosphate-buffered saline (PBS). The sample was incubated for 3 min on ice and washed 3 times with 1 mL of PBS on ice. We lysed the cells with 200 μL of SDS sample buffer. After that, the cell lysate was homogenized, sonicated using the ultrasonic homogenizer (UH-50, SMT company, Tokyo, Japan), and then boiled at 95 °C for 5 min.

In the improved method for extraction of proteins from the cells cultured on a collagen gel, we removed the medium from the cell sample and added 500 μL of 10% TCA in PBS. Then, the sample was incubated for 3 min on ice and washed 3 times with 1 mL of PBS on ice. Next, we added 250 μL (half a volume of the collagen gel) of 0.1% collagenase-L (Nitta Gelatin) in PBS to the sample and incubated the mixture at 37 °C for 1 h to allow for digestion of the collagen gel. The cells were collected into microtubes and centrifuged at 14,000 revolutions per

minute (17,700 *g*) for 3 min at 4 °C. After removing the supernatant, we lysed the cells with 200 μL of SDS sample buffer. After that, the cell lysate was homogenized, sonicated using the ultrasonic homogenizer (UH-50, SMT company, Tokyo, Japan), and then boiled at 95 °C for 5 min.

We used 10% polyacrylamide gels for SDS-PAGE (20 mA per gel, 1 h). We applied the same volume (4 μL as usual) of cell lysates to wells of the polyacrylamide gel in order to compare the protein expression among the cell lysates. After SDS-PAGE, blotting to polyvinylidene difluoride membranes was performed (92 mA per gel, 30 min for p65 and GAPDH, 60 min for integrin β1 and cyclin E1). After the blotting, the membranes were incubated in 5% skim milk in Tris-buffered saline plus Tween 20 (TBS-T) for 1 h at room temperature. The membranes were incubated with primary antibodies (p65 1:1,000, GAPDH 1:1,000,000–1:10,000,000, integrin β1 1:10,000, and cyclin E1 1:100,000) for 1 h at room temperature and/or overnight at 4 °C. After 3 washes with TBS-T, the membranes were incubated with secondary antibodies (HRP-conjugated anti-rabbit IgG antibody, 1:10,000 dilution for p65; HRP-conjugated anti-mouse IgG antibody, 1:200,000 dilution, for GAPDH; and HRP-conjugated anti-mouse IgG antibody, 1:10,000 dilution, for integrin β1 and cyclin E1) for 1 h at room temperature. Signals were detected with Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA). The relative expression of the proteins was assessed by means of the Image J software (National Institutes of Health, USA). Statistical analysis was performed using Student's or Welch's *t* test.

Results and discussion

First, we examined the expression of p65 and GAPDH proteins in the cells on a collagen gel as a soft substrate and that on a plastic dish as a stiff substrate in the presence of serum, by means of WB using the conventional method for preparing cell extracts (Fig. 1a). Extracts of the cells cultured in a plastic dish and on a collagen gel using this method are labeled in the figure as P and G respectively. GAPDH served as an internal control. The intensity of p65 bands in P was higher than in G (Fig. 1b). We elucidated whether this difference is due to the different level of p65 expression when the cells are on different substrates or due to the experimental artifacts related to the collagen gel. To this end, we added a collagen gel to the cell lysate prepared from the cells on a plastic dish. The collagen gel was preincubated with serum-containing media to mimic the collagen gel in cell culture with serum-containing media and to assess the effects of serum in the gel on the efficiency of protein detection. This condition was abbreviated as P-G (Fig. 1a). The p65 band of condition P showed higher intensity compared to P-G (Fig. 1b). These results indicated that collagen gels preincubated with serum-containing media worsened p65 detection and made it impossible to compare the expression of p65 in the cells on a collagen gel as a soft substrate with that on a plastic dish as a stiff substrate by means of WB.

To avoid the artificial deterioration of p65 detection in the cells cultured on a collagen gel, we improved the method of cell extraction by introducing trichloroacetic acid (TCA) fixation and collagenase treatment (Fig. 2a). We tested whether the improved method eliminates the problems with p65 detection caused by the collagen gel. At the same time, the effect of serum in a collagen gel on p65 detection was examined. We prepared 3 protein extracts from the cells on a plastic dish cultured with serum-containing media. We prepared the following samples: P′(+): protein extraction without a collagen gel, P′-G′(+/+): extraction when a collagen gel was added along with preincubation with serum-containing media, and P′-G′(+/-): extraction when a

collagen gel was added, but preincubation with serum-free media. P′(+) was implemented using the improved method for protein extraction from the cells cultured on a plastic dish (shown as P′ in Fig. 2a) whereas P′-G′(+/+) and P′-G′(+/-) were implemented using the method for protein extraction from the cells on a plastic dish with a collagen gel added later (shown as P′-G′ in Fig. 2a). The WB results indicated that P′(+) displayed more intense p65 bands than did P′-G′(+/+), whereas P′(+) showed almost the same intensity of the p65 bands compared to P′-G′(+/-) (Fig. 2b). These results indicated that we enabled detection of p65 bands without losses in the cells cultured with a serum-free collagen gel when we used the improved method. In contrast, p65 detection was compromised in the cells cultured with a collagen gel preincubated with serum-containing media when we used the improved method. Coomassie brilliant blue staining revealed that the P′-G′(+/+) resulted in remarkable intensity of the protein bands, whose molecular weight was 50–75 kDa, whereas the other approaches did not (Fig. 3). This protein may be albumin, which is a 66 kDa protein and a major component of serum (Peters 1977). Thus, albumin may interfere with p65 detection by blocking the reaction between the antibody and p65 as an antigen.

Next, we compared the conventional method with the improved method in the detection of proteins in A549 cells to assess the possible advantages of the improved method. In both types of samples (plastic dish and collagen gel), the improved method enhanced the detection of p65 compared to the conventional method when cells were cultured in the presence of serum (Fig. 4). Under serum-free conditions, the improved method enhanced the detection of p65 in the samples from cells grown on a collagen gel (Fig. 4). Furthermore, detection of integrin β1 and cyclin E1 was also bettered by the improved method in the samples from cells grown on a collagen gel, except for integrin β1 in the cells grown under serum-free conditions (Fig. 4). These results demonstrated that the improved method enhances protein detection in A549 cells

grown in a plastic dish in the presence of serum or on a collagen gel either with or without serum.

For further confirmation of the advantage of the improved method, we attempted to detect p65 and GAPDH in different batches of A549 cells using the conventional or the improved method. We prepared cell extracts from HT1080 human fibrosarcoma cells, NIH-3T3 mouse fibroblast cells, and mouse primary fibroblasts. Just like in A549 cells, p65 detection was enhanced by the improved method in those other cell lines when they were cultured in a plastic dish and on a collagen gel in the presence of serum (Fig. 5). Under serum-free conditions, because NIH-3T3 cells and mouse primary fibroblasts could barely adhere to a plastic dish and a collagen gel, we could not detect distinctive bands of p65 and GAPDH. Overall, in several types of cells, the improved method resulted in more visible bands of p65 compared to the conventional method.

Our results suggest that the detection of p65 is impeded by a collagen gel and serum. Thus, the p65 bands—from the A549 and HT1080 cells grown on a collagen gel under serum-free conditions—were more visible after the improved method, which can exclude a collagen gel from lysates (Fig. 4 and 5). Therefore, a collagen gel impedes the detection of p65 in western blotting. In addition, we prepared lysates of A549 and HT1080 cells grown in a plastic dish in the presence of serum using the conventional method (which cannot exclude serum proteins from lysates; Fig. 6). This approach resulted in weaker bands of p65 compared to lysates of the cells grown in a plastic dish under serum-free conditions (Fig. 4 and 5). These results suggest that serum hampers p65 detection. Thus, removal of collagen gel and serum is important for sensitive detection of p65 proteins (without losses).

In contrast to p65 detection, GAPDH detection was almost the same for the lysates prepared according to the conventional and the improved method, in each culture condition.

This may be because the collagen gel and serum affect protein detection depending on the properties of a protein under study, such as molecular weight. Indeed, molecular weight of GAPDH (approximately 37 kDa) is substantially less than that of p65 (approximately 65 kDa). Thus, detection of a protein may be affected by a collagen gel and serum, depending on the protein's molecular weight.

Next, we examined the necessity of TCA fixation and collagenase treatment in the improved method for the lossless protein detection in the cells cultured on a collagen gel under serum-free conditions. We compared the following samples: a sample treated with collagenase after TCA fixation to a sample without TCA fixation and collagenase treatment, a sample treated only with TCA, and a sample treated only with collagenase. The former showed stronger intensity of the p65 bands, although the differences were not significant (Fig. 7). Thus, both TCA fixation and collagenase treatment were necessary for the protein detection without losses in the improved method.

Finally, using the improved method, we compared the expression of the p65 protein in A549 cells cultured with serum-free media on a plastic dish with that on a collagen gel. The expression level of p65 protein was almost the same (Fig. 8). This result suggested that the difference in substrate stiffness does not affect the expression of the p65 protein in A549 cells in serum-free culture.

We showed here that the method including TCA fixation and collagenase treatment improved protein detection. In previous studies, conventional cell lysis methods, which do not involve TCA fixation and collagenase treatment, were often used for western blotting (Engler et al. 2006; Tsutsumi et al. 2009; Wu et al. 2009). In these studies, the cells were cultured on polyacrylamide gels, plastic plates, or dishes. Under these culture conditions, conventional methods may not be particularly problematic because there is almost no effect of collagen gels

on protein detection. In contrast, in our experiments, including cell culture on a collagen gel, the improved method is essential for sensitive detection of specific proteins such as p65, integrin β1, and cyclin E1 without losses caused by collagens. In western blotting analysis, our improved method is a powerful way to detect proteins in cells cultured with a collagen gel.

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Figure Captions

Fig. 1

In the conventional method, a collagen gel interferes with detection of the p65 protein in human lung adenocarcinoma A549 cells cultured on a serum-containing collagen gel. (a) A conventional procedure of preparing the protein extracts from the cells cultured on a plastic dish or on a collagen gel. The cells were cultured with serum-containing media. P: protein extraction from the cells cultured on a plastic dish. G: extraction from the cells cultured on a collagen gel. P-G: extraction from the cells cultured on a plastic dish: a collagen gel was added to the extraction to assess the artificial deterioration of the protein detection because of the collagen gel. In the P-G method, the collagen gel was preincubated with a serum-containing medium to evaluate the effect of serum in the gel on the protein detection. (b) Western blotting results for p65 and GAPDH in the samples prepared using the conventional procedure shown in (a). Serum in cell culture (+): the cells were cultured with a serum-containing medium. Serum in added gel (+): a P-G sample means that a collagen gel was added along with preincubation with a serum-containing medium. The graph shows relative intensity of p65 bands from 3 independent experiments. The data are presented as mean ± SE; $*p < 0.05$ according to Welch's *t* test

Fig. 2

An improved method allows us to detect the p65 protein without losses during protein extraction from lung adenocarcinoma A549 cells cultured on a collagen gel under serum-free conditions. (a) An improved procedure of preparing the extracts from the cells cultured on a plastic dish or on a collagen gel. The added steps in the improved procedure are highlighted in red. P′: protein extraction from the cells cultured on a plastic dish. G′: extraction from the cells cultured on a collagen gel. P′-G′: extraction from the cells cultured on a plastic dish, where a collagen gel was added to the samples before collagenase treatment. (b) GAPDH and p65 data from western blotting after the P′ and P′-G′ methods where the samples were prepared using the improved procedure shown in (a). Serum in cell culture (+): the cells were cultured with serum-containing media. Serum in added gel (+): P′-G′ means that a collagen gel was added before collagenase treatment, and the collagen gel was preincubated with a serum-containing medium. Serum in added gel (-): P′-G′ means that a collagen gel was added before collagenase treatment, and the collagen gel was preincubated with a serum-free medium. The graph shows the relative intensity of p65 bands from 3 independent experiments. The data are presented as mean ± SE; $*p < 0.05$ according to Welch's *t* test

Fig. 3

The P′-G′ sample (where we added a collagen gel preincubated with a serum-containing medium) shows a protein band of remarkable intensity, whose molecular weight was 50–75 kDa, detected by means of Coomassie brilliant blue staining. The Coomassie brilliant blue staining results in the P′ and P′-G′ samples prepared using the improved procedure shown in Fig. 2a. P′: protein extraction from the cells cultured on a plastic dish. P′-G′: extraction from the cells cultured on a plastic dish, where a collagen gel was added to the samples before collagenase treatment. Serum in cell culture (+): the cells were cultured with serum-containing media. Serum in added gel (+): the P′-G′ sample where we added a collagen gel before collagenase treatment, and the collagen gel was preincubated with a serum-containing medium. Serum in the added gel (-): the P′-G′ sample where we added a collagen gel before collagenase treatment, and the collagen gel was preincubated with a serum-free medium. A representative result from 3 independent experiments is shown

Fig. 4

Detection of p65, integrin β1, and cyclin E1 is enhanced by the improved method in A549 cells cultured in a plastic dish and on a collagen gel. The figure shows western blotting data on p65, integrin β1, cyclin E1, and GAPDH after protein extraction from the cells cultured in a plastic dish (P) or on a collagen gel (G). Serum in cell culture (+): the cells were cultured in serum-containing media. Serum in cell culture (-): the cells were cultured in serum-free media. Method (C): extraction according to the conventional method shown in Fig. 1a. Method (I): extraction according to the improved method shown in Fig. 2a. The graph shows relative intensity of p65, integrin β1, and cyclin E1 bands from 3 independent experiments. The data are presented as mean ± SE; $*p < 0.05$ according to Student's or Welch's *t* test

Fig. 5

Detection of p65 is enhanced by the improved method in various types of cells cultured in a plastic dish and on a collagen gel. The figure shows western blotting data on p65 and GAPDH after protein extraction from the cells cultured in a plastic dish (P) or on a collagen gel (G). Serum in cell culture (+): the cells were cultured in serum-containing media. Serum in cell culture (-): the cells were cultured in serum-free media. Method (C): extraction according to the conventional method shown in Fig. 1a. Method (I): extraction according to the improved method shown in Fig. 2a. HT: human fibrosarcoma HT1080 cells, 3T3: mouse fibroblast NIH-3T3 cells, MP: mouse primary fibroblasts. The graph shows relative intensity of p65 bands in each cell type from 3 independent experiments. The data are presented as mean ± SE, $*p < 0.05$ according to Student's or Welch's *t* test

Fig. 6

The lysate (of A549 cells grown in a plastic dish in the presence of serum) prepared using the conventional method shows a protein band of remarkable intensity, whose molecular weight was 50–75 kDa, detected by means of Coomassie brilliant blue staining. The Coomassie brilliant blue staining results in lysates from the cells cultured in a plastic dish (P). Serum in cell culture (+): the cells were cultured in serum-containing media. Serum in cell culture (-): the cells were cultured in serum-free media. Method (C): extraction according to the conventional method shown in Fig. 1a. Method (I): extraction according to the improved method shown in Fig. 2a. A representative result from 3 independent experiments is shown

Fig. 7

TCA fixation and collagenase treatment are necessary for p65 protein detection without losses in the improved method shown in Fig. 2a. Western blotting data on p65 and GAPDH after protein extraction from the cells cultured on a collagen gel. Serum in cell culture (-): the cells were cultured with serum-free media. TCA (+): the cells fixed with 10% TCA and washed 3 times before protein extraction. TCA (-): the cells without TCA fixation and after washes before extraction. Collagenase (+): the cells treated with 0.1% collagenase after removal of the collagen gel before protein extraction. Collagenase (-): the cells without collagenase treatment after removal of the collagen gel before extraction. The graph shows relative intensity of p65 bands from 3 independent experiments. The data are presented as mean ± SE

Fig. 8

There are no significant differences in p65 expression between lung adenocarcinoma A549 cells cultured in a plastic dish and the same cells cultured on a collagen gel under serum-free conditions. Western blotting data on p65 and GAPDH after protein extraction—according to the improved method shown in Fig. 2a—from the cells cultured in a plastic dish (P′) or on a collagen gel (G′). Serum in cell culture (-): the cells were cultured in serum-free media. The graph shows relative intensity of p65 bands from 3 independent experiments. The data are presented as mean ± SE

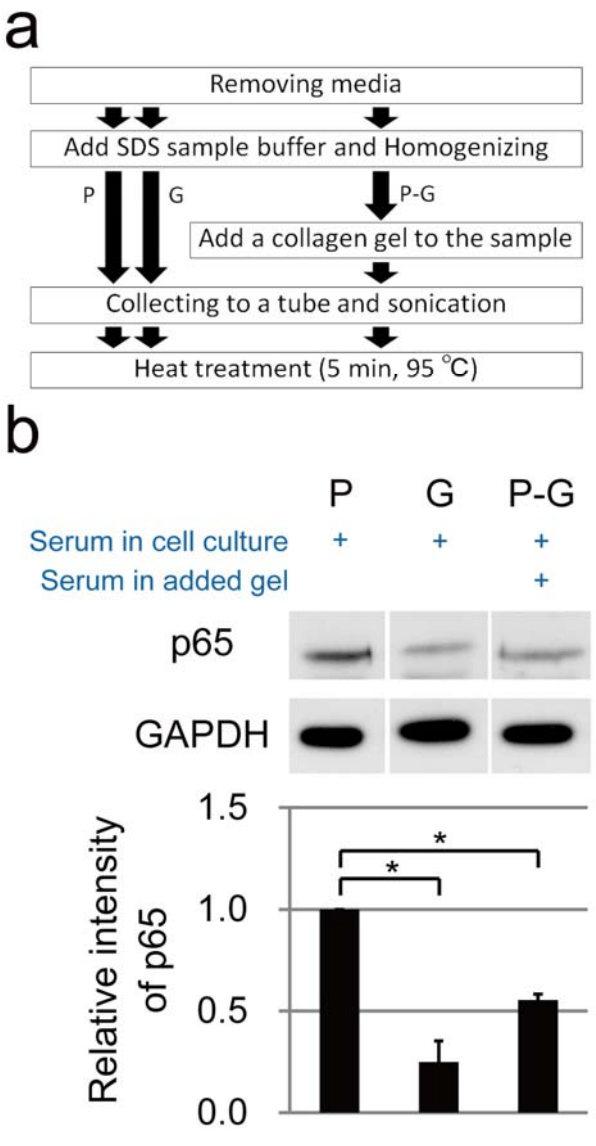
a
Removing media
Add SDS sample buffer and Homogenizing
P
G
P-G
Add a collagen gel to the sample
Collecting to a tube and sonication
Heat treatment (5 min, 95 °C)
b
P
G
P-G
Serum in cell culture
+
+
+
Serum in added gel
+
p65
GAPDH
Relative intensity of p65
1.5
1.0
0.5
0.0
*
*

Fig. 1

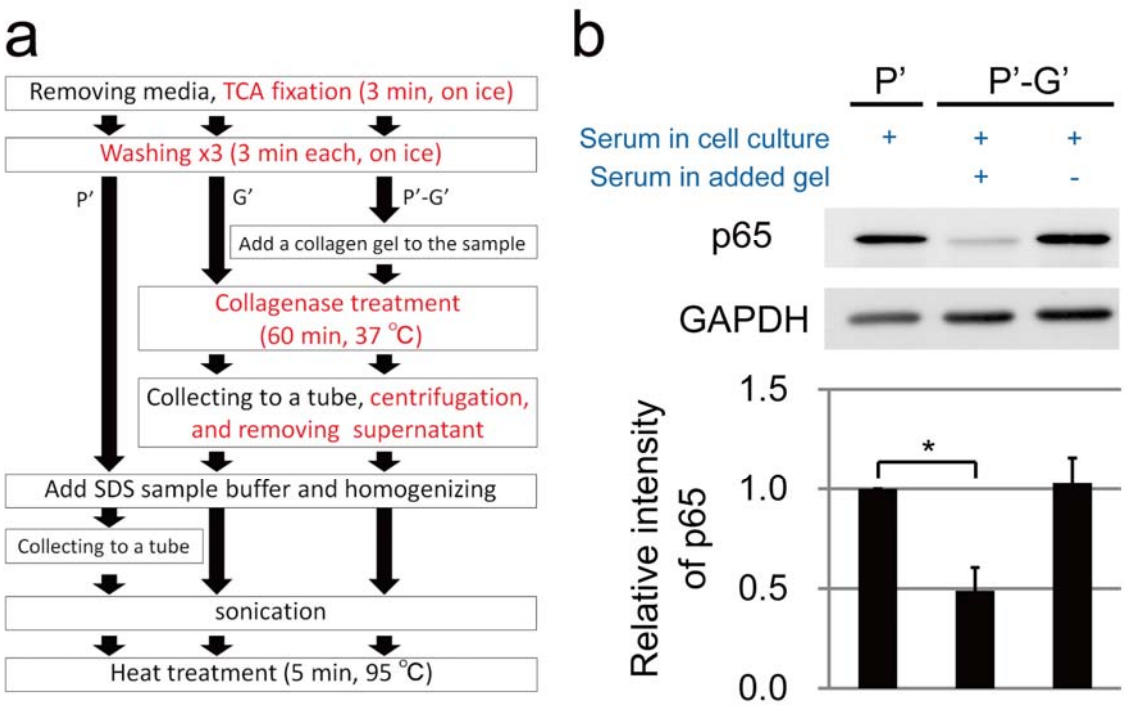
a
Removing media, TCA fixation (3 min, on ice)
Washing x3 (3 min each, on ice)
P'
G'
P'-G'
Add a collagen gel to the sample
Collagenase treatment
(60 min, 37 °C)
Collecting to a tube, centrifugation,
and removing supernatant
Add SDS sample buffer and homogenizing
Collecting to a tube
sonication
Heat treatment (5 min, 95 °C)
b
P'
P'-G'
Serum in cell culture
+
+
+
Serum in added gel
+
-
p65
GAPDH
Relative intensity
of p65
1.5
1.0
0.5
0.0
*

Fig. 2

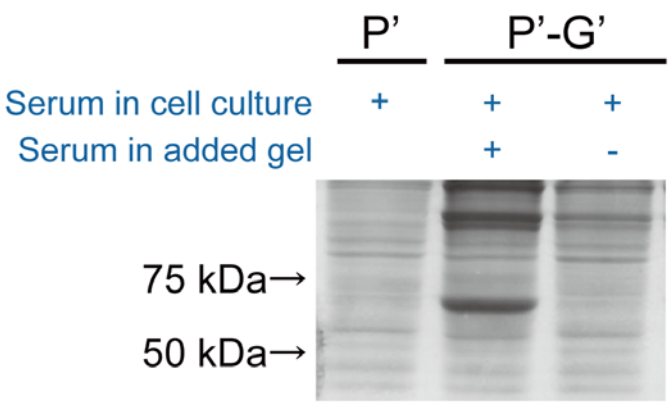

Fig. 3

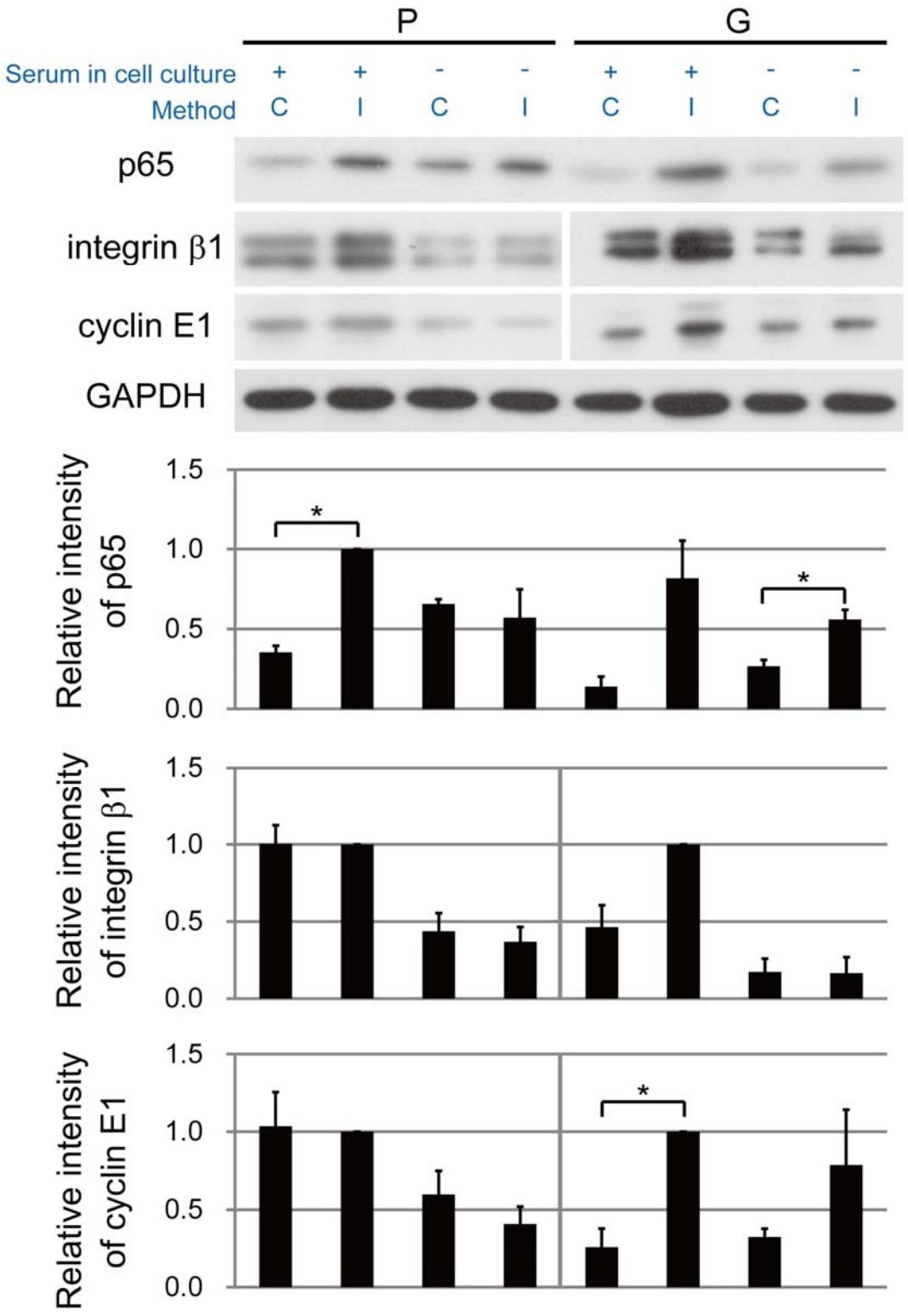
P
G
Serum in cell culture + + - - + + - -
Method C I C I C I C I
p65
integrin β1
cyclin E1
GAPDH
Relative intensity of p65
Relative intensity of integrin β1
Relative intensity of cyclin E1
1.5
1.0
0.5
0.0
*
*
*

Fig. 4

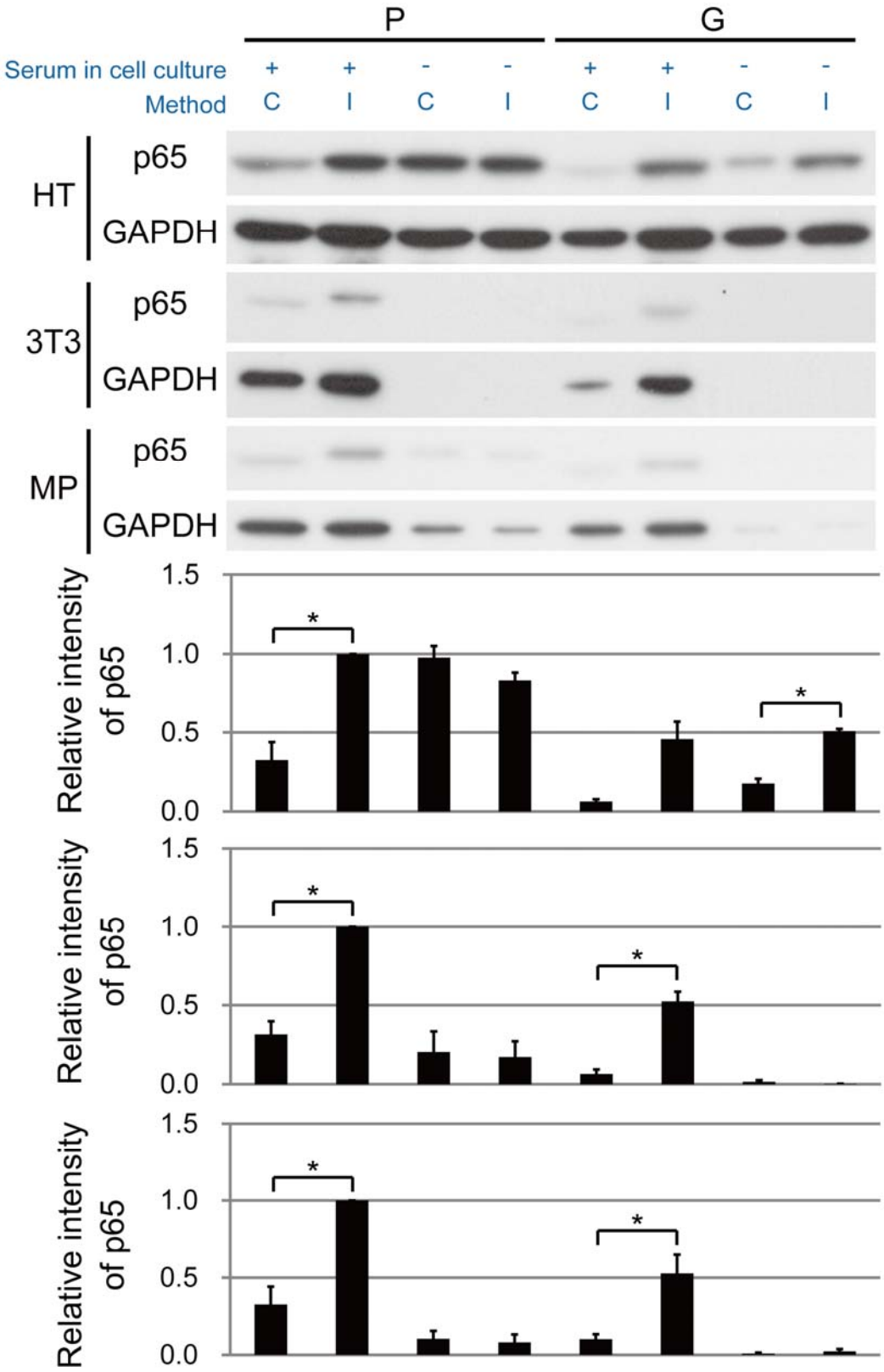
P
G
Serum in cell culture + + - - + + - -
Method C I C I C I C I
HT
p65
GAPDH
3T3
p65
GAPDH
MP
p65
GAPDH
Relative intensity of p65
1.5
1.0
0.5
0.0
*
Relative intensity of p65
1.5
1.0
0.5
0.0
*
Relative intensity of p65
1.5
1.0
0.5
0.0
*

Fig. 5

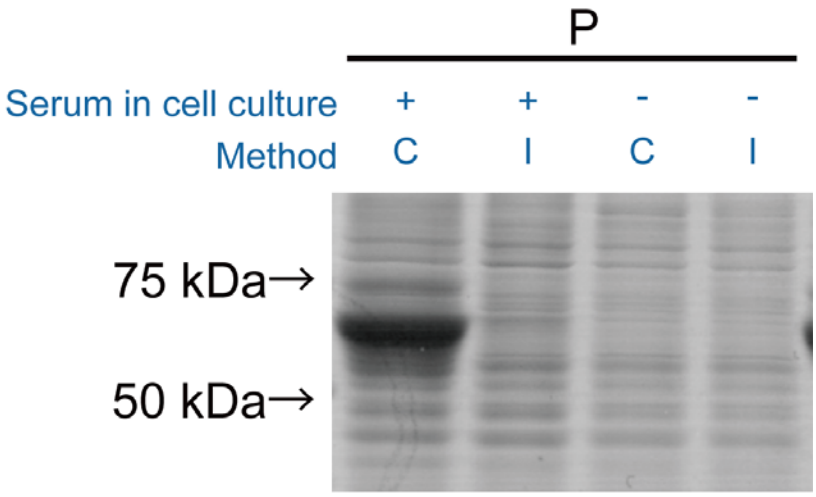

Fig. 6

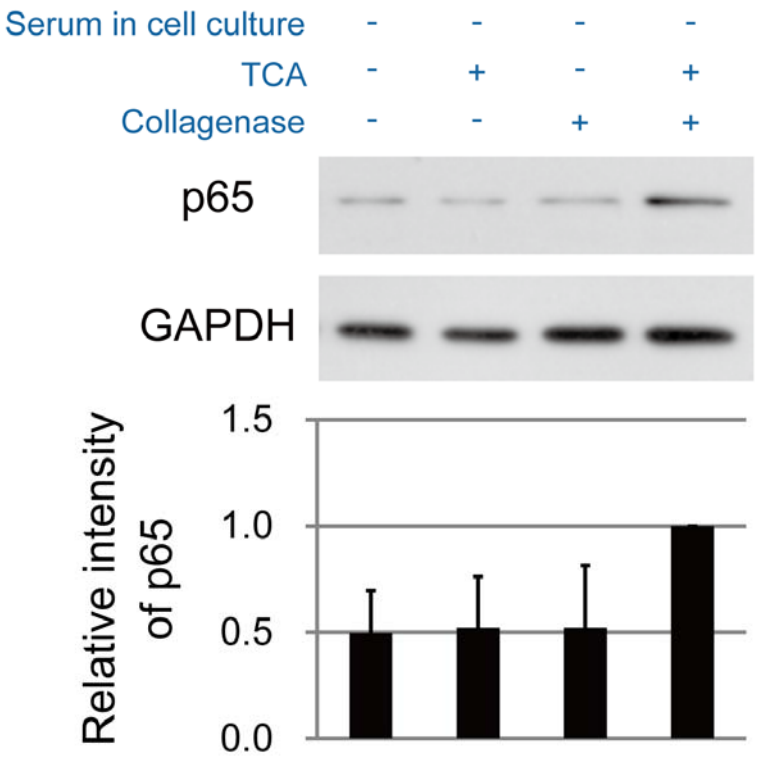
Serum in cell culture - - - -
TCA - + - +
Collagenase - - + +
p65
GAPDH
Relative intensity of p65
1.5
1.0
0.5
0.0

Fig. 7

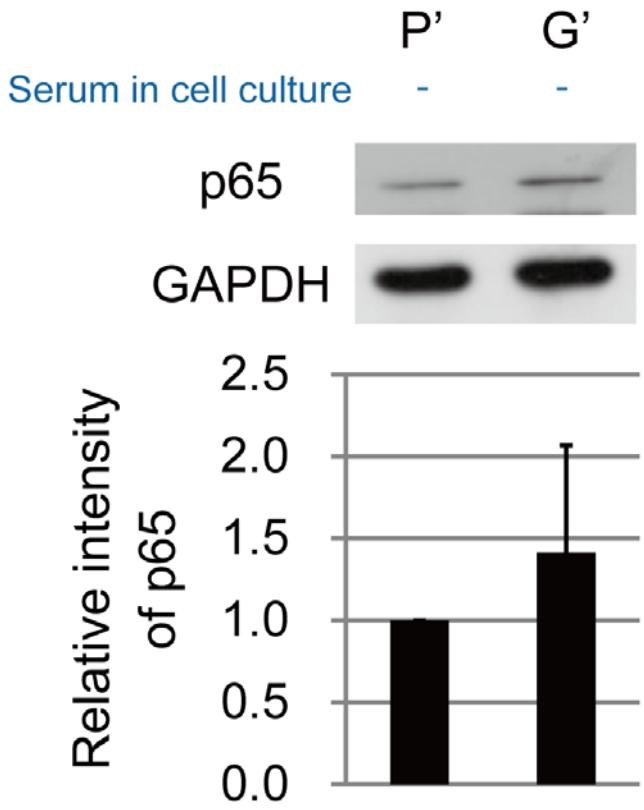
P'
G'
Serum in cell culture
-
-
p65
GAPDH
Relative intensity of p65
2.5
2.0
1.5
1.0
0.5
0.0

Fig. 8