肺胞上皮細胞のフラジェリン刺激による上皮成長因子受容体のセリン1047のリン酸化は、MAPキナーゼ活性化プロテインキナーゼ2によって起こる

Phosphorylation of epidermal growth factor receptor at serine 1047 by MAP kinase-activated protein kinase-2 in cultured lung epithelial cells treated with flagellin

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Short Title: Phosphorylation of EGFR by MAPKAPK-2

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Abbreviations: CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; MAP kinase, mitogen-activated protein kinase; MAPKAPK-2, mitogen-activated protein kinase-activated protein kinase-2; TLR5, Toll-like receptor 5; TGF-β1, transforming growth factor beta 1; TNFα, tumor necrosis factor α.

Nomenclature: ERK1/2, also called p44/p41 mitogen-activated protein kinase or MAPK3/MAPK1; MAPKAPK-2, also called MK2; MEK, also called MAP2K or MAPKK; p38 MAP kinase, also called CSBP, MAPK14 or RK.

Abstract

It has been reported that tumor necrosis factor α (TNFα) activated the p38 MAP kinase pathway, followed by phosphorylation of epidermal growth factor receptor (EGFR) at serine 1047 (Ser1047). Although the phosphorylation of Ser1047 reportedly induced an internalization of EGFR, a protein kinase responsible for the phosphorylation has not been elucidated. In the present study, we found that treatment with flagellin of A549 cells, an alveolar epithelial cell line, induced the activation of p38 MAP kinase, followed by phosphorylation of EGFR at Ser1047. The phosphorylation was strongly inhibited by SB203580, an inhibitor of p38 MAP kinase. The flagellin treatment activated MAP kinase-activated protein kinase-2 (MAPKAPK-2), a protein kinase downstream of p38 MAP kinase, and MK2a inhibitor, an inhibitor of MAPKAPK-2, inhibited the flagellin-induced phosphorylation of EGFR at Ser1047. Unlike the flagellin treatment, the TNFα treatment induced the phosphorylation of EGFR at both Ser1047 and Tyr1173. SB203580 and MK2a inhibitor strongly inhibited the phosphorylation of Ser1047 but not Tyr1173 in EGFR. Finally, bacterially expressed and activated MAPKAPK-2 phosphorylated EGFR at Ser1047 *in vitro*. These results strongly suggested that MAPKAPK-2 directly phosphorylated EGFR at Ser1047 after treatment of A549 cells with flagellin and TNFα.

Keywords: Alveolar epithelial cells; A549 cells; EGFR; Flagellin; MAPKAPK-2; p38 MAP kinase.

When epidermal growth factor (EGF) binds to the EGF receptor (EGFR), multiple tyrosine residues are autophosphorylated, resulting in the initiation of multiple signal transduction pathways (for review, see [1]). At the same time, the EGF-occupied EGFR is rapidly internalized by receptor-mediated endocytosis [2]. The internalized EGFR is sorted to lysosomes where it is degraded. Tumor necrosis factor α (TNFα), a cytokine especially important in inducing inflammatory responses, activates the nuclear factor-κB (NF-κB) signaling pathway (for review, see [3]). It was reported that TNFα transactivated EGFR through soluble transforming growth factor α (TGFα) in the AML-12 hepatocyte cell line [4]. The transactivation mechanisms involved a cell surface metalloproteinase that released TGFα from pro-TGFα anchored in the plasma membrane [4]. In addition to these effects, TNFα reportedly activated the p38 MAP kinase pathway, and induced the transient phosphorylation of EGFR at serine 1047 (Ser1047) [1]. The phosphorylated EGFR was internalized through a clathrin-mediated mechanism [5]. It was interesting that EGFR phosphorylated at Ser1047 was not degraded at lysosomes, and that inactivation of the p38 MAP kinase pathway led to dephosphorylation of EGFR and its recycling back to the cell surface. However, a protein kinase that is activated by the p38 MAP kinase pathway and directly phosphorylates EGFR at Ser1047 has remained to be elucidated.

Flagellin, a primary structural component of bacterial flagella, binds to and activates Toll-like receptor 5 (TLR5) in vertebrates [6]. TLR5 reportedly recognized 13 amino acid residues in flagellin, which are buried and are not accessible in polymerized flagellar filaments [7]. It is well known that stimulation of TLR5 by flagellin activates the gene expression of pro-inflammatory cytokines such as interleukin-8 in intestinal

epithelial cells [8] (for review, see [9]). However, the pathophysiological roles of TLR5 stimulation in pulmonary infections by flagellated bacteria, such as *Legionella pneumophila*, are not clear at present.

A549 cells are immortalized alveolar epithelial cells, and this cell line has been used as a model to study the responses of alveolar epithelial cells to several treatments [10, 11]. *Legionella pneumophila* was reportedly able to replicate within A549 cells [12]. Recently, we found that flagellin treatment induced long-term activation of the p38 MAP kinase pathway (Kondo et al., unpublished observations).

In the present study, we found that treatment of A549 cells with flagellin induced the phosphorylation of EGFR at Ser1047, and that MAP kinase-activated protein kinase-2 (MAPKAPK-2) was responsible for the phosphorylation. In addition, we found that MAPKAPK-2 was involved also in the TNFα-induced phosphorylation of EGFR at Ser1047. However, there were several differences in the phosphorylation of EGFR between flagellin and TNFα treatments.

Materials and Methods

Materials

The following chemicals and reagents were obtained from the indicated sources: [γ-^{32}P]ATP, PerkinElmer Japan Co. (Tokyo, Japan); Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS), Sigma Chemical Co. (St Louis, MO); fetal calf serum (FCS), HyClone (Logan, UT); DynaMarker Protein MultiColor, BioDynamics Laboratory Inc. (Tokyo, Japan); U0126, anti-MAPKAPK-2 antibody, anti-phospho-MAPKAPK-2 (Thr222) rabbit monoclonal antibody (9A7), anti-phospho-MAPKAPK-2 (Thr334) rabbit monoclonal antibody (27B7), anti-p38 MAP kinase antibody, and anti-phospho-p38 MAP kinase antibody, Cell Signaling Technology (Beverly, MA); KN93, anti-ERK antibody, Sigma Chemical Co. (Saint Louis, MO); anti-active ERK antibody, Promega Co. (Madison, WI); anti-EGFR mouse monoclonal antibody (6F1)(IgG_{2b}), Assay designs (Ann Arbor, MI); anti-multi ubiquitin mouse monoclonal antibody (FK2), MBL Co. (Nagoya, Japan); anti-phospho-EGFR (Thr669) mouse monoclonal antibody (5F10)(anti-P-Thr669 EGFR antibody) and anti-phospho-EGFR (Ser1047) mouse monoclonal antibody (1H9) (anti-P-Ser1047 EGFR antibody), Acris Antibodies Inc. (San Diego, CA); protease inhibitor cocktail (PI cocktail) and phosphatase inhibitor cocktail (EDTA free) (PPI cocktail), Nacalai Tesque (Kyoto, Japan); anti-phospho-EGFR (Tyr1173) mouse monoclonal antibody (9H2)(anti-P-Tyr1173 EGFR antibody), AG1478 [4-(3-Chloroanilino)-6,7-dimethoxyquinazoline], SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole], and MK2a inhibitor [4-(2'-fluorobiphenyl-4-yl)-N-(4-hydroxyphenyl)-butyramide], Calbiochem

(Darmstadt, Germany); syntide-2, Bachem Americas, Inc. (Torrance, CA); purified flagellin from *Bacillus subtilis*, InvivoGen (San Diego, CA); TNFα, Wako (Osaka, Japan); and recombinant human transforming growth factor beta 1 (TGF-β1), R&D Systems Inc. (Minneapolis, MN). Other chemicals were of analytical grade.

Culture of A549 cells and preparation of cell extracts

A549 cells were grown on Petri dishes (Nunc, Roskilde, Denmark) in a culture medium consisting of DMEM containing 4.5 g/L of glucose and 10% (vol/vol) heat-inactivated FCS as described previously [3]. We chose the concentrations of inhibitors of signal transduction (U0126, KN93, AG1478, SB203580, and MK2a inhibitor) as directed by the manufacturer's instructions. A549 cells on 60-mm Petri dishes were washed once in PBS and lysed in 300 μl of 1 x SDS-PAGE sample buffer containing 2% (wt/vol) SDS, 62.5 mM Tris-HCl, pH 6.8, 5% (vol/vol) 2-mercaptoethanol, 5% (vol/vol) glycerol, and 0.01% (wt/vol) bromophenol blue [13, 14]. The cell extract was sonicated for 20 sec on ice, treated at 98°C for 5 min, and kept at -80°C prior to use.

SDS-PAGE and immunoblot analysis

SDS-PAGE was performed by the method of Laemmli [14], followed by an immunoblot analysis [15, 16]. Immunoreactive proteins were detected using the enhanced chemiluminescence detection kit (GE Healthcare UK Ltd., Little Chalfont, England) as directed by the manufacturer. Phosphorylation levels of the proteins were measured by quantification of the amounts of phosphoproteins present. For the

quantification, we used a LAS4000 mini (GE Healthcare UK Ltd.) with Multi Gauge software (version 3.1). In order to estimate the apparent molecular weights of the proteins by chemiluminescence, we loaded the MagicMark XP Western Protein Standard (Invitrogen, Carlsbad, CA) on all SDS-PAGE gels. The position of 170 kDa was estimated from 220-kDa and 120-kDa standard proteins. For reprobing, the membrane was incubated with stripping buffer [62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% (wt/vol) SDS] at 50°C for 30 min [16]. The membrane was then washed with a blocking solution containing 5% (wt/vol) skim milk, 100 mM Tris-HCl, pH 7.5, 0.9% (wt/vol) NaCl, and 0.1% (vol/vol) Tween-20, and Tris-buffered saline with Tween-20 (TTBS) containing 100 mM Tris-HCl, pH 7.5, 0.9% (wt/vol) NaCl, and 0.1% (vol/vol) Tween-20 at room temperature and subjected to an immunoblot analysis.

Immunoprecipitation

A549 cells were lysed on ice in homogenization buffer containing 60 mM Tris-HCl, pH 7.5, 180 mM NaCl, 6 mM EDTA, 2 mM EGTA, 18 mM $Na_4P_2O_7$, 60 mM NaF, 1.2% (vol/vol) Nonidet P-40, 1mM dithiothreitol, PI cocktail and PPI cocktail. We diluted the PI cocktail and PPI cocktail as directed by the manufacturer's instructions. The homogenate was centrifuged at 13,000 *g* for 5 min at 4°C to obtain the supernatant (cell extract). The cell extract was pre-cleared by a 20-min incubation at 4ºC with mouse control serum and protein A Sepharose CL-4B (Amersham Bioscience, Uppsala, Sweden), followed by centrifugation at 14,000 *g* for 2 min at 4°C. The supernatant was incubated at 4ºC for 2.5 h with anti-EGFR monoclonal antibody or mouse control serum.

The antibody was immobilized on 50% (vol/vol) protein A Sepharose CL-4B with shaking at 4ºC overnight. The immunoprecipitate was eluted from protein A Sepharose CL-4B by adding SDS-PAGE sample buffer, boiling for 2 min, and centrifugation at 14,000 *g* for 2 min [17]. The eluate was subjected to SDS-PAGE in 7.5% (wt/vol) acrylamide, and an immunoblot analysis was performed with anti-multi ubiquitin antibody (FK2) or anti-EGFR antibody as described above.

Purification and activation of glutathione S-transferase-fusion MAPKAPK-2

The cDNA of MAPKAPK-2 was obtained from mouse kidney and sequenced. The characterization of the cDNA will be described elsewhere. Glutathionine *S*-transferase (GST)-fused MAPKAPK-2 (GST-MAPKAPK-2) was expressed in *Escherichia coli* strain BL21, and purified using glutathione-Sepharose beads (GE Healthcare UK Ltd.) as described previously [17]. GST-MAPKAPK-2 was dialyzed against 10 mM Tris-HCl, pH 8.0, and 10% (vol/vol) glycerol at 4°C and kept at -80°C. GST-MAPKAPK-2 was activated by phosphorylation with extracellular signal-regulated kinase (ERK) as described [18]. GST-MAPKAPK-2 (5 μg) was incubated with activated-ERK (0.4 U)(Sigma Chemical Co.) at 30°C for 60 min in the presence of 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 0.2 mM EGTA and 0.2 mM ATP. The activated GST-MAPKAPK-2 was immediately used for phosphorylation of syntide-2 or EGFR.

Assay for MAPKAPK-2

The assay system for MAPKAPK-2 contained the following constituents: 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 0.2 mM EGTA, 0.1 mM [γ-^{32}P]ATP (3,000-5,000

cpm/pmol), 40 μM syntide-2 and activated GST-MAPKAPK-2 (1.5 μM). After 10-min incubation, the samples were spotted on phosphocellulose paper squares and the amount of phosphate incorporated into syntide-2 was determined as described [19].

Overexpression of EGFR in HEK293T cells and its in vitro phosphorylation

HEK293T cells were grown on 60-mm Petri dishes (Nunc, Roskilde, Denmark) in a culture medium consisting of DMEM containing 4.5 g/L of glucose, 10% (vol/vol) heat-inactivated FCS, 50 U/ml penicillin and 50 μg/ml streptomycin. The expression vector for human EGFR (pCAGI Puro-EGFR) was kindly provided by Dr. H. Nakanishi of Kumamoto University. The cells were transfected with pCAGI Puro-EGFR (4.8 μg of plasmid DNA) using 7.5 μl of FuGENE HD transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) in 5 ml of standard medium. Then, they were further cultured for 24 h. The cells were lysed on ice in homogenization buffer containing 60 mM Tris-HCl, pH 7.5, 180 mM NaCl, 6 mM EDTA, 1.2% (vol/vol) Nonidet P-40 and PI cocktail. Immunoprecipitation of EGFR was performed as described above. After centrifugation at 13,000 *g* for 5 min, the pellets were washed four times with 0.01% (wt/vol) BSA, 100 mM Tris-HCl, pH 7.5 and 0.9% (wt/vol) NaCl as described previously [20]. Immunoprecipitates were phosphorylated at 30°C for 30 min in the presence of 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mM EGTA and 0.1 mM ATP by GST-MAPKAPK-2 which was pre-activated as described above. EGFR was eluted from protein A Sepharose CL-4B by adding 2 x SDS-PAGE sample buffer, boiling for 2 min, and centrifugation at 14,000 *g* for 5 min. The eluate was subjected to SDS-PAGE

in 7.5% (wt/vol) acrylamide, and an immunoblot analysis of EGFR with anti-P-Ser1047 EGFR antibody and anti-EGFR antibody was performed.

Other procedures

Protein concentrations were determined using the Qubit Protein Assay kit with the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). In pilot experiments, the protein concentration curves were linear in the presence of 0.04% (wt/vol) SDS, 0.1% (vol/vol) 2-mercaptoethanol and 0.1% (vol/vol) glycerol. Therefore, we diluted the cell extract 50 fold with water for protein quantification. The experiments were repeated at least three times and representative results are shown in figures. Values are expressed as the mean ± SE. The statistical analysis was performed using a one-way ANOVA plus Duncan's multiple range test. $P < 0.05$ was considered statistically significant.

Results

Phosphorylation of EGFR at Ser1047 by flagellin

We first examined whether or not phosphorylation of EGFR was increased by the flagellin treatment in A549 cells (Fig. 1). The phosphorylation of EGFR at Ser1047 was increased approximately 3.2 and 14.6 fold by flagellin at 0.2 and 2 μg/ml, respectively, compared with the control (Fig. 1A). In contrast, no increase in phosphorylation at Tyr1173 was observed, suggesting that flagellin did not activate EGFR. When the protein level of EGFR was examined, no significant changes by flagellin were observed. We noticed that the electrophoretic mobility of EGFR decreased slightly after treatment of the cells with 2 μg/ml flagellin. However, this decrease was not observed in repeated experiments (e.g. Fig. 1B).

We treated A549 cells with 2 μg/ml flagellin for various periods (Fig. 1B). The phosphorylation increased approximately 18 fold at 30 and 90 min, compared with the control. The phosphorylation was sustained, and an approximately 17-fold increase was still observed at 120 min.

Involvement of p38 MAP kinase in flagellin-induced phosphorylation of EGFR

Recently, we found that flagellin strongly activated the p38 MAP kinase pathway in A549 cells (Kondo et al., unpublished observations). In Fig. 1B, we compared the time course of the phosphorylation of p38 MAP kinase with that of the phosphorylation of EGFR at Ser1047. Phosphorylation of p38 MAP kinase was increased approximately 6 fold at 15 min compared with the control, and peaked at 30 min. Phosphorylation of p38 MAP kinase was increased approximately 21 and

12 fold at 30 and 90 min, respectively. These results indicated that the activation of p38 MAP kinase and phosphorylation of EGFR occurred with a similar time course. Then, we examined whether or not the phosphorylation of EGFR by flagellin was inhibited by SB203580, a specific inhibitor of p38 MAP kinase (Fig. 1C). Interestingly, phosphorylation at Ser1047 was abolished by SB203580. These results indicated that the p38 MAP kinase pathway was necessary for the phosphorylation of EGFR at Ser1047.

Activation by flagellin of MAPKAPK-2 and its involvement in EGFR phosphorylation

Next, we attempted to identify the protein kinase which directly phosphorylated EGFR at Ser1047 following the flagellin treatment. P38 MAP kinase belongs to the proline-directed protein kinases (for review, see [18]). The primary amino acid sequence around Ser1047 is RYSSDP, and Ser1047 is followed by an aspartic acid residue; therefore, we speculated that Ser1047 was not directly phosphorylated by p38 MAP kinase. MAPKAPK-2 is a protein kinase downstream of p38 MAP kinase, and is activated by multisite phosphorylation by p38 MAP kinase [21]. One physiological substrate for MAPKAPK-2 is heat shock protein 27 (HSP27) (for review, see [18]). HSP27 is phosphorylated at Ser78 and Ser82, both of which occur in the sequence motif RXXS [22]. We noticed that Ser1047 in EGFR is also in the RXXS motif. Therefore, we considered the possibility that MAPKAPK-2 might directly phosphorylate EGFR at Ser1047 following flagellin treatment. We first examined whether or not MAPKAPK-2 was

activated by flagellin treatment. The mutational analysis of MAPKAPK-2 suggested that phosphorylation of any two of the residues Thr222, Ser272 and Thr334 was necessary and sufficient for maximal activation of MAPKAPK-2 [18]. Conversely, it was reported that the phosphorylation of any one residue was unable to achieve significant activation [18]. In the present experiment, we examined the phosphorylation of Thr222 and Thr334, because we could not obtain a specific antibody against MAPKAPK-2 phosphorylated at Ser272. In A549 cells treated with 2 μg/ml flagellin for various periods, the phosphorylation of Thr334 was robustly increased at 15 min, peaking at 30 min and declining thereafter (Fig. 2A). In contrast, phosphorylation at Thr222 was only weakly detected at 30 min and increased gradually until 120 min. Assuming that Ser272 was not phosphorylated by the flagellin treatment, these results would suggest that MAPKAPK-2 was activated from 30 min. Immunoblot analysis with an anti-MAPKAPK-2 antibody showed that the electrophoretic mobility of MAPKAPK-2 decreased at 15 min, and this decrease was sustained until 120 min, suggesting that phosphorylation of MAPKAPK-2 by p38 MAP kinase decreased the electrophoretic mobility.

As MAPKAPK-2 was activated by flagellin, we examined whether it was involved in the phosphorylation of EGFR. MK2a inhibitor, which binds to MAPKAPK-2 and prevents its activation, inhibited the flagellin-induced phosphorylation of EGFR at 20 μM (Fig. 2B). The results from three independent experiments are summarized in Fig. 2C. The phosphorylation of EGFR in the absence of flagellin was 19.3 ± 1.5% of that in its presence ($P < 0.01$). MK2a inhibitor inhibited the phosphorylation by 78.0 ± 3.6% ($P < 0.01$) at 20 μM. These

results strongly suggested that MAPKAPK-2 was responsible for the phosphorylation of Ser1047 following flagellin treatment. We noticed a concomitant decrease in the phosphorylation of MAPKAPK-2 at Thr 222 and Thr334 (Fig. 2B). The change in electrophoretic mobility was not clear in this experiment.

Phosphorylation of EGFR at Ser1047 and Tyr1173 by TNFα

We next examined whether MAPKAPK-2 was also responsible for the phosphorylation of Ser1047 by TNFα. First, we confirmed the phosphorylation of EGFR by TNFα under our experimental conditions (Fig. 3). Previously, we found that the optimal concentration of TNFα for the activation of the NF-κB pathway was 100 ng/ml [3]. Therefore, we treated A549 cells with 100 ng/ml TNFα for various periods (Fig. 3A). Phosphorylation of EGFR at Ser1047 was first detected at 5 min, peaked at 15 min, and declined at 30 min. The phosphorylation of Ser1047 by TNFα was more pronounced than that by flagellin, and increased approximately 100 and 78 fold at 15 and 30 min, respectively. We found that the phosphorylation of Tyr1173 increased approximately 12 fold at 15 min, declining at 30 min, suggesting that TNFα treatment transiently activated EGFR.

In addition, we noticed that the decrease in electrophoretic mobility was more pronounced with TNFα than flagellin (Fig. 3A). It has been reported that the TNFα treatment of HeLa cells decreased the electrophoretic mobility of EGFR [5, 23]. We first considered the decrease to be due to the autophosphorylation of tyrosine residues. AG1478, a relatively specific inhibitor of EGFR tyrosine kinase, inhibited

the phosphorylation of Tyr1173 (Fig. 3B). However, the decrease in mobility was not affected in the presence of AG1478. Ubiquitylation of EGFR has been well studied in other cell types (for review, see [24]). To examine whether the decrease in electrophoretic mobility was due to ubiquitylation, we immunoprecipitated EGFR after TNFα treatment (Fig. 3C). Immunoblot analysis with an anti-EGFR antibody clearly showed a decrease in mobility, but no ubiquitylation of EGFR. These results indicated that the mobility shift of EGFR was not due to autophosphorylation or ubiquitylation.

Activation of ERK and p38 MAP kinase by TNFα

Next, we examined whether ERK or p38 MAP kinase directly phosphorylated EGFR to induce the mobility shift after TNFα treatment. Activation of ERK and p38 MAP kinase was first detected at 5 min, peaked at 15 min, and declined at 30 min (Fig. 4A). The time courses were similar to that for the mobility shift of EGFR (Fig. 3A). The primary amino acid sequence around Thr669 is EPLTPSG, and it was reported that Thr669 was directly phosphorylated by ERK and p38 MAP kinase [1, 25, 26]. It was reported that treatment of A549 cells with TNFα induced phosphorylation of EGFR at Thr669 [1]. We confirmed that EGFR was phosphorylated at Thr669 after treatment of A431 cells with both PMA and pervanadate (Fig. 4B). However, Thr669 was not phosphorylated at all by the treatment of A549 cells with TNFα. The reasons for the difference from the previous report [1, 25, 26] are not clear at present. These results strongly suggested

that the mobility shift of EGFR was not due to direct phosphorylation at Thr669 by ERK or p38 MAP kinase.

Inhibition by SB203580 of phosphorylation of EGFR and MAPKAPK-2

We examined whether or not the phosphorylation of EGFR by TNFα was inhibited by SB203580 (Fig. 5A). We found that the phosphorylation of Ser1047 was strongly inhibited by SB203580, while the phosphorylation of Tyr1173 was not inhibited at all. Surprisingly, the mobility shift was completely abolished by SB203580, suggesting that the p38 MAP kinase pathway was involved in the mobility shift by TNFα.

We next examined whether or not MAPKAPK-2 was activated by TNFα (Fig. 5B). Both Thr222 and Thr334 were strongly phosphorylated by TNFα, and a concomitant decrease in the electrophoretic mobility of MAPKAPK-2 was clearly observed. As expected, SB203580 strongly inhibited the activation and mobility shift of MAPKAPK-2.

Involvement of MAPKAPK-2 in phosphorylation of EGFR

Next, we examined whether or not MAPKAPK-2 was involved in the phosphorylation of EGFR. When the cells were pretreated with MK2a inhibitor at 2 or 20 μM, phosphorylation of EGFR at Ser1047 was inhibited in a concentration-dependent manner (Fig. 6A). The results from three independent experiments are summarized in Fig. 6B. The phosphorylation of EGFR in the absence of TNFα was 1.6 ± 0.7% of that in its presence ($P < 0.001$). MK2a

inhibitor inhibited the phosphorylation of EGFR by 64.6 ± 4.0% ($P < 0.01$) and 92.4 ± 1.3% ($P < 0.01$) at 2 and 20 μM, respectively. The difference between cells treated with TNFα and 2 μM MK2a inhibitor and those treated with TNFα and 20 μM MK2a inhibitor was statistically significant ($P < 0.01$).

Phosphorylation of MAPKAPK-2 at Thr222 and Thr334 was slightly inhibited by MK2a inhibitor at 2 μM, and strongly inhibited at 20 μM (Fig. 6A). However, the recovery of the mobility shift of EGFR and MAPKAPK-2 was not complete even at 20 μM of MK2a inhibitor.

Effects of U0126 and KN93 on the phosphorylation of EGFR at Ser1047

An *in-vitro* experiment revealed that ERK phosphorylated and activated MAPKAPK-2 as well as p38 MAP kinase [18]. Therefore, we examined whether or not U0126, a MEK inhibitor, inhibited the TNFα-induced phosphorylation of EGFR at Ser1047 (Fig. 7A). We found that U0126 inhibited by about 30% the phosphorylation of EGFR. These results may suggest that ERK is involved in the phosphorylation of EGFR as well as p38 MAP kinase.

It has been reported that Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) phosphorylated EGFR at Ser1047 following the stimulation of EGFR in CHO cells expressing EGFR [27]. However, the TNFα-induced phosphorylation of EGFR at Ser1047 was not inhibited by KN93, an inhibitor of CaM kinase II (Fig. 7A). These results indicated that CaM kinase II was not involved in the TNFα-induced phosphorylation of EGFR at Ser1047 in A549 cells. Immunoblot

analysis with anti-EGFR antibody showed that the amount of EGFR protein was not changed by any treatment (Fig. 7A).

Effects of TGF-β1 on the phosphorylation of EGFR at Ser1047

It has been reported that TGF-β1 activated the p38 MAP kinase pathway in pulmonary epithelial 1HAEo$^-$ cells [28]. Recently, we confirmed that p38 MAP kinase was activated by the treatment of A549 cells with TGF-β1 (Kondo et al., unpublished observations). We found that the activation of p38 MAP kinase by TGF-β1 at 10 ng/ml was comparable with that by flagellin at 2 μg/ml (Kondo et al., unpublished observations). Therefore, we examined whether or not TGF-β1 activated MAPKAPK-2 and induced the phosphorylation of EGFR at Ser1047 (Fig. 7B). It was interesting that the phosphorylation of EGFR by TGF-β1 was much weaker than that by flagellin. The phosphorylation of EGFR at Ser1047 by TGF-β1 at 120 min was less than 10% of that by flagellin at 30 min. These results suggested that not all activators of p38 MAP kinase induced the phosphorylation of EGFR at Ser1047. Immunoblot analysis with anti-EGFR antibody showed that the amount of EGFR protein was not changed by any treatment (Fig. 7B). Phosphorylation of MAPKAPK-2 at Thr334 was not detected by TGF-β1, suggesting that MAPKAPK-2 was not activated by the TGF-β1 treatment. However, the electrophoretic mobility of some MAPKAPK-2 decreased by the TGF-β1 treatment and its two protein bands were observed (Fig. 7B). These results may suggest that some other sites than Thr334 were phosphorylated by TGF-β1.

Phosphorylation of EGFR at Ser1047 in vitro by MAPKAPK-2

Our results indicated that MAPKAPK-2 was involved in the phosphorylation of EGFR at Ser1047 after treatment with flagellin and TNFα. We then examined whether MAPKAPK-2 could directly phosphorylate EGFR at Ser1047 *in vitro*. To this end, we bacterially overexpressed and purified GST-MAPKAPK-2. It was reported that bacterially overexpressed GST-MAPKAPK-2 was inactive and could be activated by ERK through phosphorylation of Thr222, Ser272 and Thr334 [18]. That study reported also that syntide-2 was a good substrate for MAPKAPK-2 *in vitro* [18]. Immunoblot analysis of GST-MAPKAPK-2 showed that both Thr222 and Thr334 were phosphorylated *in vitro* by activated ERK (Fig. 8A). Furthermore, we measured the activity of GST-MAPKAPK-2 with syntide-2 and [γ-^{32}P]ATP. When GST-MAPKAPK-2 was used without its pre-phosphorylation by ERK, only 6.0 pmol of phosphate were incorporated into syntide-2. In contrast, 763.2 pmol of phosphate were incorporated after its pre-phosphorylation by ERK. We also confirmed that only 5.6 pmol of phosphate were incorporated by ERK alone. These results clearly indicated that GST-MAPKAPK-2 could be activated by ERK *in vitro*.

We then overexpressed EGFR in HEK293T cells and immunoprecipitated it with a monoclonal antibody to the carboxy-terminal of EGFR, followed by phosphorylation with MAPKAPK-2 pre-phosphorylated by ERK (Fig. 8B). Immunoblot analysis showed that Ser1047 was weakly phosphorylated in the presence of ERK without MAPKAPK-2. These results may suggest that ERK could phosphorylate EGFR at Ser1047. Furthermore, the phosphorylation of EGFR

at Ser1047 was enhanced in the presence of GST-MAPKAPK-2 pre-phosphorylated by ERK. These results indicated that GST-MAPKAPK-2 could phosphorylate EGFR at Ser1047 *in vitro*.

Discussion

The experiments with MK2a inhibitor clearly indicated that MAPKAPK-2 was involved in the phosphorylation of EGFR at Ser1047 in A549 cells treated with flagellin and TNFα (Figs. 2 and 6). We then confirmed that purified MAPKAPK-2 could phosphorylate EGFR at Ser1047 *in vitro* (Fig. 8). These results strongly suggested that MAPKAPK-2 directly phosphorylated EGFR at Ser1047. We found that the phosphorylation by TNFα was transient, but more pronounced than that by flagellin (Figs. 1-3 and 6). These results may suggest that the molecular mechanisms for the activation of p38 MAP kinase and MAPKAPK-2 differ between TNFα and flagellin treatments. It is also possible that some protein kinases downstream from MAPKAPK-2 phosphorylated EGFR at Ser1047 after the flagellin or TNFα treatment. To our knowledge, this is the first report of the activation of MAPKAPK-2 by flagellin.

It has been reported that EGFR is phosphorylated at Ser1047 and internalized after TNFα treatment [5], suggesting that cell surface EGFR is phosphorylated by MAPKAPK-2. Recently, we found that MAPKAPK-2 was located mainly in the cytosolic fraction (Noguchi et al, unpublished observations). Therefore, MAPKAPK-2 may translocate to the membrane from the cytosol in order to phosphorylate cell surface EGFR. The translocation of MAPKAPK-2 after flagellin and TNFα treatments is worth examining in a future study.

We noticed that the mobility shift of EGFR was more pronounced with TNFα than with flagellin (Figs. 1 and 3). A p38 MAP kinase inhibitor (SB203580) completely inhibited the shift, but MK2a inhibitor only partially inhibited it (Figs.

5 and 6). We confirmed that both inhibitors almost completely prevented the phosphorylation of EGFR at Ser1047. These results suggest that p38 MAP kinase activated by TNFα activates some protein kinases other than MAPKAPK-2 and induces the phosphorylation of EGFR at multiple sites, although Thr669 was definitely not phosphorylated (Fig. 4).

We confirmed that EGFR was phosphorylated at Tyr1173 by TNFα, suggesting that EGFR was activated, as reported in cultured hepatocytes (AML-12cells) [4] (Fig. 3). However, phosphorylation of Tyr1173 was not increased after flagellin treatment (Fig. 1). These results may suggest that TLR5's stimulation by flagellin induced the down-regulation of EGFR without the activation of EGFR. The pathophysiological roles of the phosphorylation of EGFR in pulmonary infections by flagellated bacteria are not clear at present. Recently, we found that the stimulation of TLR5 by flagellin induced the epithelial-mesenchymal transition (EMT) in A549 cells (Kondo et al., unpublished observations). Therefore, it is worth examining whether or not phosphorylation and down-regulation of EGFR are involved in the induction of EMT.

Legionella pneumophila causes Legionnaire pneumonia in susceptible individuals [29]. It has been suggested that TLR5's stimulation by flagellin has a critical role in the onset of this disease [29]. Legionella is phagocytosed by alveolar macrophages, and a cellular immune response leads to acute alveolitis with an exudate, which is rich in alveolar macrophages. Because Legionella replicates intracellularly in alveolar macrophages, it is highly possible that Legionella is abundantly released in alveolar spaces together with macrophages, and then

macrophages and Legionella are killed in the local inflammatory space. In that case, it is reasonable to speculate that flagellin protofilaments, which are released from macrophages or derived from polymerized flagella in the killed bacteria, robustly increase in alveolar spaces to stimulate TLR5 in surviving alveolar epithelial cells, leading to phosphorylation of EGFR at Ser1047. Idiopathic pulmonary fibrosis is a progressive disease characterized by the accumulation of fibroblasts, which leads to disruption of the alveolar architecture, and a decline in lung function [30]. It is not clear at present whether phosphorylation of EGFR at Ser1047 is involved in this pulmonary fibrosis. Recently, it has been reported that activation of ERK following stimulation of EGFR promoted renal fibrosis [31]. Activation of the p38 MAP kinase and phosphorylation of EGFR at Ser1047 may modulate these effects of EGFR stimulation.

In the present study, we used flagellin purified from *Bacillus subtilis* to stimulate TLR5. Because amino acids crucial for TLR5's recognition are conserved both in *Bacillus subtilis* and in *Legionella pneumophila* [7, 32], it is highly possible that flagellin from *Legionella pneumophila*, as well as *Bacillus subtilis*, stimulates TLR5 to phosphorylate EGFR at Ser1047 in alveolar epithelial cells.

Acknowledgments

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FIGURE LEGENDS

Fig. 1. Phosphorylation of EGFR and activation of p38 MAP kinase by flagellin. A, A549 cells were treated with flagellin at the indicated concentrations for 30 min. The cell extracts (51 μg) were subjected to SDS-PAGE in 7.5% (wt/vol) acrylamide, and an immunoblot analysis was performed with anti-P-Ser1047 EGFR antibody (1: 100) or anti-P-Tyr1173 EGFR antibody (1: 300). After the anti-P-Ser1047 EGFR antibody was stripped away, immunoblotting with anti-EGFR antibody (1: 600) was performed. B, A549 cells were treated with 2 μg/ml of flagellin for the periods indicated. An immunoblot analysis of P-Ser1047 EGFR and EGFR was performed using the cell extracts (43.5 μg) as described above. The cell extracts (43.5 μg) were also subjected to SDS-PAGE in 10% (wt/vol) acrylamide, and an immunoblot analysis was performed with anti-phospho-p38 MAP kinase antibody (1: 750). After the antibody was stripped away, immunoblotting with p38 MAP kinase antibody was performed at a dilution of 1: 750. C, A549 cells were pretreated with or without 5 μM SB203580 for 30 min and treated with or without 2 μg/ml of flagellin for 30 min. The cell extracts

(67.5 μg) were subjected to SDS-PAGE and an immunoblot analysis of P-Ser1047 EGFR and EGFR was performed as described above. The bands for EGFR (P-Ser1047 EGFR, P-Tyr1173 EGFR and EGFR) and p38 MAP kinase (P-p38 MAP kinase and p38 MAP kinase) are indicated.

Fig. 2. Activation by flagellin of MAPKAPK-2 and its involvement in phosphorylation of EGFR at Ser1047. A, A549 cells were treated with 2 μg/ml of flagellin for the periods indicated. The cell extracts (43.5 μg) were subjected to SDS-PAGE in 10% (wt/vol) acrylamide and an immunoblot analysis was performed with anti-P-Thr222 MAPKAPK-2 antibody (1: 750) or anti-P-Thr334 MAPKAPK-2 antibody (1: 750). After the anti-P-Thr222 MAPKAPK-2 antibody was stripped away, immunoblotting with anti-MAPKAPK-2 antibody was performed at a dilution of 1: 750. B, A549 cells were pretreated with or without 2 or 20 μM MK2a inhibitor (MK2ai) for 60 min and treated with or without 2 μg/ml of flagellin for 30 min. The cell extracts (33 μg) were subjected to SDS-PAGE in 7.5% (wt/vol) acrylamide and an immunoblot analysis of P-Ser1047 EGFR and EGFR was performed as described in the legend for Fig. 1. The cell extracts (33 μg) were also subjected to SDS-PAGE in 10% (wt/vol) acrylamide and an immunoblot analysis was performed with anti-P-Thr222 MAPKAPK-2 antibody (1: 750) or anti-P-Thr334 MAPKAPK-2 antibody (1: 750). After the anti-P-Thr334 MAPKAPK-2 antibody was stripped away, immunoblotting with anti-MAPKAPK-2 antibody was performed at a dilution of 1: 750. The bands for MAPKAPK-2 (P-Thr222 MAPKAPK2, P-Thr334 MAPKAPK2 and MAPKAPK2)

and EGFR (P-Ser1047 EGFR and EGFR) are indicated. C, The phosphorylation of EGFR at Ser1047 in the presence of flagellin was taken as 100%, and the other values were calculated relative to this. Values are the mean ± SE (three samples per condition). $^{**}P < 0.01$ (*vs*. control). The difference between cells treated with flagellin and those treated with both flagellin and 20 μM MK2a inhibitor was statistically significant ($^{\#\#}P < 0.01$).

Fig. 3. Phosphorylation of EGFR by TNFα. A, A549 cells were treated with 100 ng/ml of TNFα for the periods indicated. The cell extracts (24 μg) were subjected to SDS-PAGE in 7.5% (wt/vol) acrylamide, and an immunoblot analysis was performed with anti-P-Ser1047 EGFR antibody (1: 100), anti-P-Tyr1173 EGFR antibody (1: 300) or anti-EGFR antibody (1: 600). B, A549 cells were pretreated with or without 1 μM AG1478 for 60 min and treated with or without 100 ng/ml of TNFα for 15 min. The cell extracts (31.5 μg) were subjected to SDS-PAGE in 7.5% (wt/vol) acrylamide, and an immunoblot analysis was performed with anti-EGFR antibody (1: 600) or anti-P-Tyr1173 EGFR antibody (1: 300). The bands for EGFR (P-Ser1047 EGFR, P-Tyr1173 EGFR and EGFR) are indicated. C, A549 cells were treated with or without 100 ng/ml of TNFα for 15 min. Immunoprecipitation was done with anti-EGFR antibody, followed by an immunoblot analysis with anti-multi ubiquitin antibody (1: 500)(Ub) as described in the Materials and Methods. After the antibody was stripped away, immunoblotting with anti-EGFR antibody was performed at a dilution of 1: 600 (EGFR). The arrows indicate the bands for EGFR.

Fig. 4. Activation of ERK and p38 MAP kinase by TNFα. A, A549 cells were treated with 100 ng/ml of TNFα for the periods indicated. The cell extracts (24 μg) were subjected to SDS-PAGE in 10% (wt/vol) acrylamide, and an immunoblot analysis was performed with anti-active ERK antibody (1: 750) or anti-phospho-p38 MAP kinase antibody (1: 750). After each antibody was stripped away, immunoblotting with anti-ERK antibody (1: 1,000) or anti-p38 MAP kinase antibody (1: 750) was performed. B, The cell extracts (24 μg) were also subjected to SDS-PAGE in 7.5% (wt/vol) acrylamide, and an immunoblot analysis was performed with anti-P-Thr669 EGFR antibody (1: 100). For the positive control, the extracts from A431 cells treated with phorbol 12-myristate 13-acetate (PMA) and pervanadate (PV)(Acris Antibodies Inc.) were loaded in the leftmost lane. The bands for ERK (P-ERK1, P-ERK2, ERK1 and ERK2), p38 MAP kinase (P-p38 MAP kinase and p38 MAP kinase) and EGFR (P-Thr669 EGFR) are indicated.

Fig. 5. Inhibition by SB203580 of TNFα-induced phosphorylation of EGFR and MAPKAPK-2. A, A549 cells were pretreated with or without 5 μM SB203580 for 30 min and treated with or without 100 ng/ml of TNFα for 15 min. The cell extracts (33 μg) were subjected to SDS-PAGE in 7.5% (wt/vol) acrylamide, and an immunoblot analysis was performed with anti-P-Ser1047 EGFR antibody (1: 100), anti-P-Tyr1173 EGFR antibody (1: 300) or anti-EGFR antibody (1: 600). B, The cell extracts (33 μg) were also subjected to SDS-PAGE in 10% (wt/vol) acrylamide, and an immunoblot analysis was performed with anti-P-Thr222 MAPKAPK-2

antibody (1: 750) or anti-P-Thr334 MAPKAPK-2 antibody (1: 750). After the anti-P-Thr334 MAPKAPK-2 antibody was stripped away, immunoblotting with anti-MAPKAPK-2 antibody (1: 1,000) was performed. The bands for EGFR (P-Ser1047 EGFR, P-Tyr1173 EGFR and EGFR) and MAPKAPK-2 (P-Thr222 MAPKAPK2, P-Thr334 MAPKAPK2, P-MAPKAPK2 and MAPKAPK2) are indicated.

Fig. 6. Inhibition by MK2a inhibitor of the phosphorylation of EGFR at Ser1047 and activation of MAPKAPK-2 by TNFα. A, A549 cells were pretreated with or without 2 or 20 μM MK2a inhibitor (MK2ai) for 60 min and treated with or without 100 ng/ml of TNFα for 15 min. The cell extracts (58.8 μg) were subjected to SDS-PAGE in 7.5% (wt/vol) acrylamide and an immunoblot analysis of P-Ser1047 EGFR and EGFR was performed as described in the legend for Fig. 1. The cell extracts (58.8 μg) were also subjected to SDS-PAGE in 10% (wt/vol) acrylamide and an immunoblot analysis was performed with anti-P-Thr222 MAPKAPK-2 antibody (1: 750) and anti-P-Thr334 MAPKAPK-2 antibody (1: 750). After the anti-P-Thr334 MAPKAPK-2 antibody was stripped away, immunoblotting with anti-MAPKAPK-2 antibody was performed at a dilution of 1: 750. The bands for EGFR (P-Ser1047 EGFR and EGFR) and MAPKAPK-2 (P-Thr222 MAPKAPK2, P-Thr334 MAPKAPK2, P-MAPKAPK2 and MAPKAPK2) are indicated. B, The phosphorylation of EGFR at Ser1047 in the presence of TNFα was taken as 100%, and the other values were calculated relative to this. Values are the mean ± SE (three samples per condition). $^{**}P < 0.01$

(*vs*. control). The difference between cells treated with TNFα and those treated with both TNFα and MK2a inhibitor (2 or 20 μM) was statistically significant ($^{\#\#}P < 0.01$).

Fig. 7. Effects of U0126 and KN93 on TNFα-induced phosphorylation of EGFR, and phosphorylation of EGFR and MAPKAPK-2 by TGF-β1. A, A549 cells were pretreated with or without 10 μM U0126 or 20 μM KN93 for 30 min and treated with or without 100 ng/ml of TNFα for 15 min. The cell extracts (22.5 μg) were subjected to SDS-PAGE in 7.5% (wt/vol) acrylamide, and an immunoblot analysis was performed with anti-P-Ser1047 EGFR antibody (1: 100). After the anti-P-Ser1047 EGFR antibody was stripped away, immunoblotting with anti-EGFR antibody (1: 600) was performed. B, A549 cells were treated with 10 ng/ml of TGF-β1 or 2 μg/ml of flagellin for the periods indicated. The cell extracts (35.3 μg) were subjected to SDS-PAGE in 7.5% (wt/vol) acrylamide, and an immunoblot analysis was performed with anti-P-Ser1047 EGFR antibody (1: 100). After the anti-P-Ser1047 EGFR antibody was stripped away, immunoblotting with anti-EGFR antibody (1: 600) was performed. The cell extracts (35.3 μg) were also subjected to SDS-PAGE in 10% (wt/vol) acrylamide, and an immunoblot analysis was performed with anti-P-Thr334 MAPKAPK-2 antibody (1: 750). After the anti-P-Thr334 MAPKAPK-2 antibody was stripped away, immunoblotting with anti-MAPKAPK-2 antibody (1: 1,000) was performed. The bands for EGFR (P-Ser1047 EGFR and EGFR) and MAPKAPK-2 (P-Thr334 MAPKAPK2, P-MAPKAPK2 and MAPKAPK2) are indicated.

Fig. 8. Phosphorylation of GST-MAPKAPK-2 by ERK, and phosphorylation by GST-MAPKAPK-2 of EGFR at Ser1047. A. Purified GST-MAPKAPK-2 (5.0 μg) was phosphorylated in the presence or absence of ERK (0.4 U) for 60 min with 0.2 mM ATP. Samples of GST-MAPKAPK-2 (2.2 μg) were subjected to SDS-PAGE in 10% (wt/vol) acrylamide and an immunoblot analysis was performed with anti-P-Thr222 MAPKAPK-2 antibody (1: 750) or anti-P-Thr334 MAPKAPK-2 antibody (1: 750). After the anti-P-Thr222 MAPKAPK-2 antibody was stripped away, immunoblotting with anti-MAPKAPK-2 antibody was performed at a dilution of 1: 750. B. EGFR was overexpressed in HEK293T cells and immunoprecipitation of EGFR was performed in the presence or absence of anti-EGFR monoclonal antibody as described in Materials and Methods. The immunoprecipitate was phosphorylated in the presence of ERK with or without the pre-activated GST-MAPKAPK-2 (GST-MAPKAPK2) for 30 min with 0.1 mM ATP. EGFR was eluted from protein A Sepharose CL-4B by treatment with 50 μl of 2 x SDS-PAGE sample buffer, samples of the eluate (25 μl) were subjected to SDS-PAGE in 7.5% (wt/vol) acrylamide, and an immunoblot analysis of P-Ser1047 EGFR and EGFR was performed as described in the legend for Fig. 1. The bands for GST-MAPKAPK-2 (P-Thr222 MAPKAPK2, P-Thr334 MAPKAPK2 and MAPKAPK2) and EGFR (P-Ser1047 EGFR and EGFR) are shown. The apparent molecular mass of GST-MAPKAPK-2 was approximately 68 kDa. Arrowheads indicate what appears to be a degradated EGFR which contained phosphorylated Ser1047.

Fig. 1

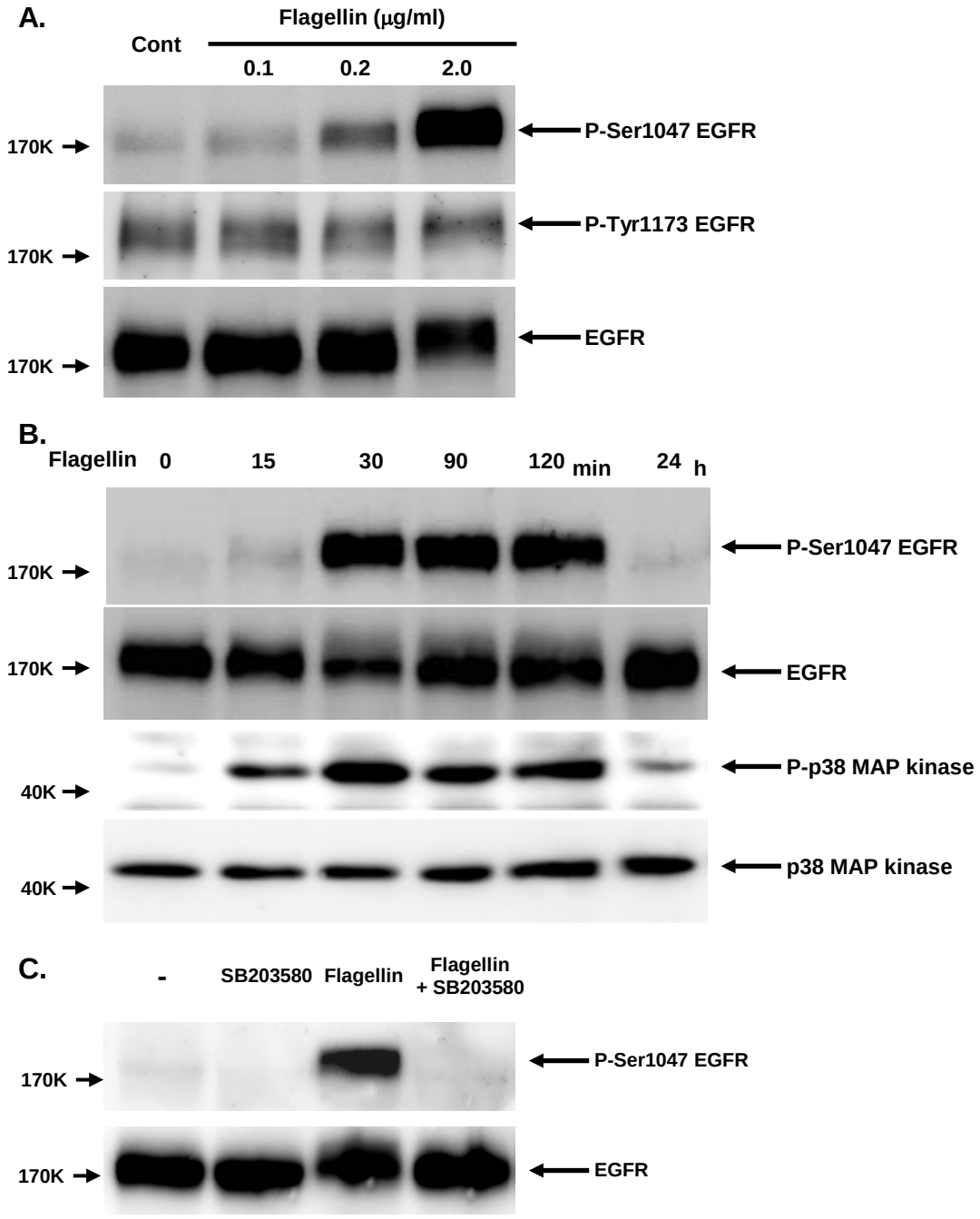

A.
Flagellin (μg/ml)
Cont
0.1
0.2
2.0
170K
P-Ser1047 EGFR
170K
P-Tyr1173 EGFR
170K
EGFR
B.
Flagellin
0
15
30
90
120 min
24 h
170K
P-Ser1047 EGFR
170K
EGFR
40K
P-p38 MAP kinase
40K
p38 MAP kinase
C.
-
SB203580
Flagellin
Flagellin
+ SB203580
170K
P-Ser1047 EGFR
170K
EGFR

Fig. 2

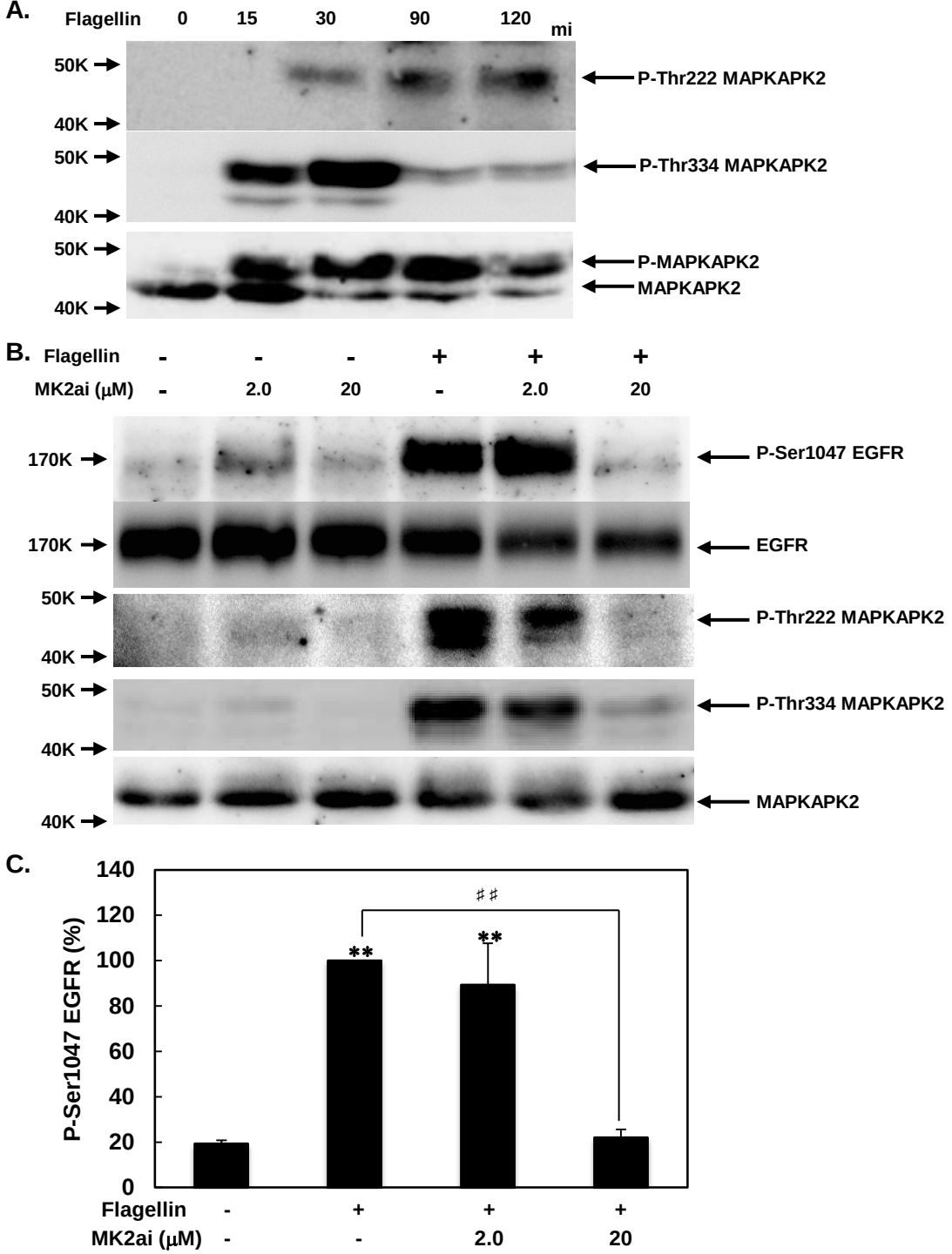

A.
Flagellin 0 15 30 90 120 mi
50K
40K
P-Thr222 MAPKAPK2
P-Thr334 MAPKAPK2
P-MAPKAPK2
MAPKAPK2
B.
Flagellin - - - + + +
MK2ai (μM) - 2.0 20 - 2.0 20
170K
P-Ser1047 EGFR
EGFR
P-Thr222 MAPKAPK2
P-Thr334 MAPKAPK2
MAPKAPK2
C.
P-Ser1047 EGFR (%)
0 20 40 60 80 100 120 140
**
##
Flagellin - + + +
MK2ai (μM) - - 2.0 20

Fig. 3

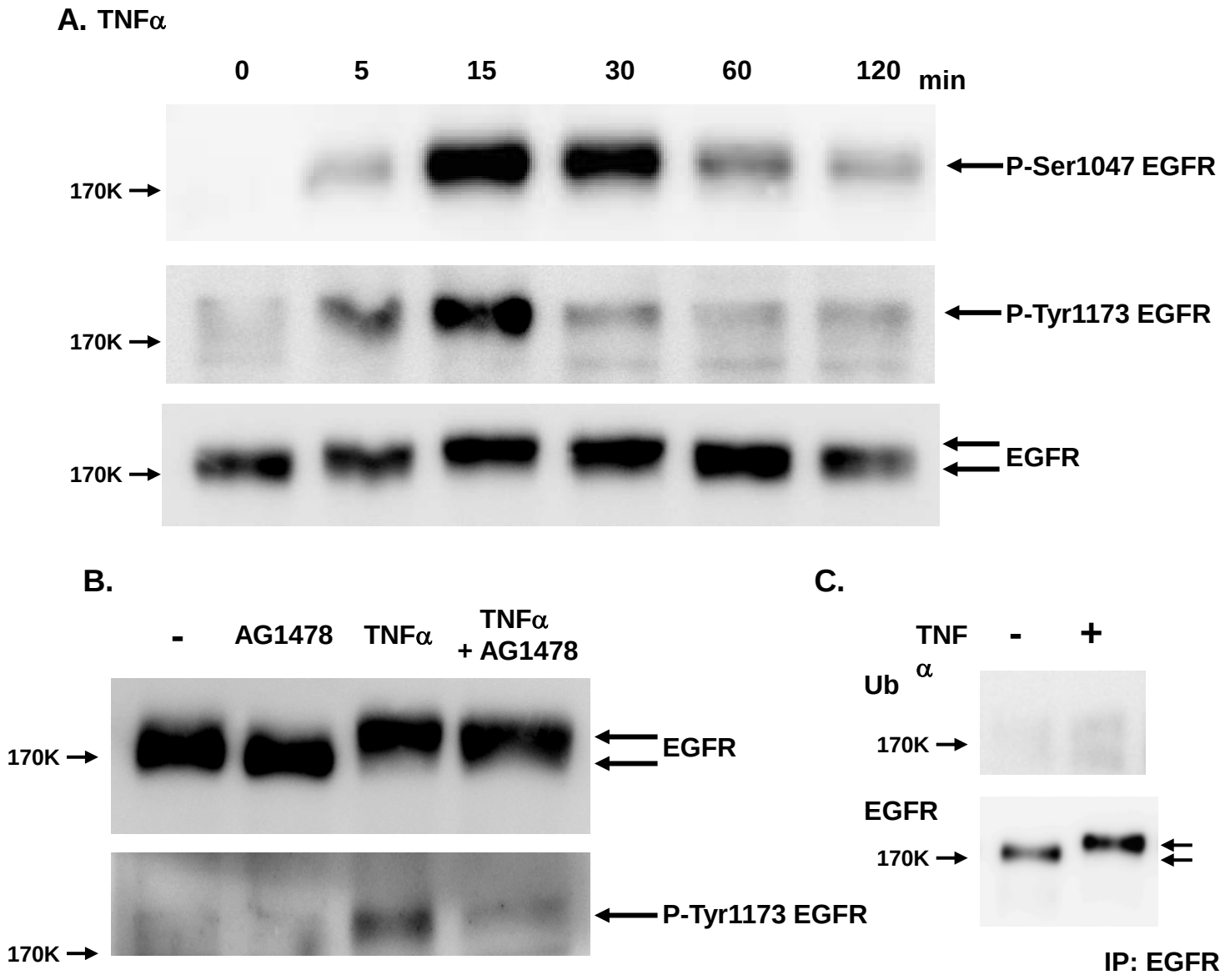

A. TNFα
0
5
15
30
60
120
min
170K
P-Ser1047 EGFR
170K
P-Tyr1173 EGFR
170K
EGFR
B.
-
AG1478
TNFα
TNFα + AG1478
170K
EGFR
170K
P-Tyr1173 EGFR
C.
TNF α
-
+
Ub
170K
EGFR
170K
IP: EGFR

Fig. 4

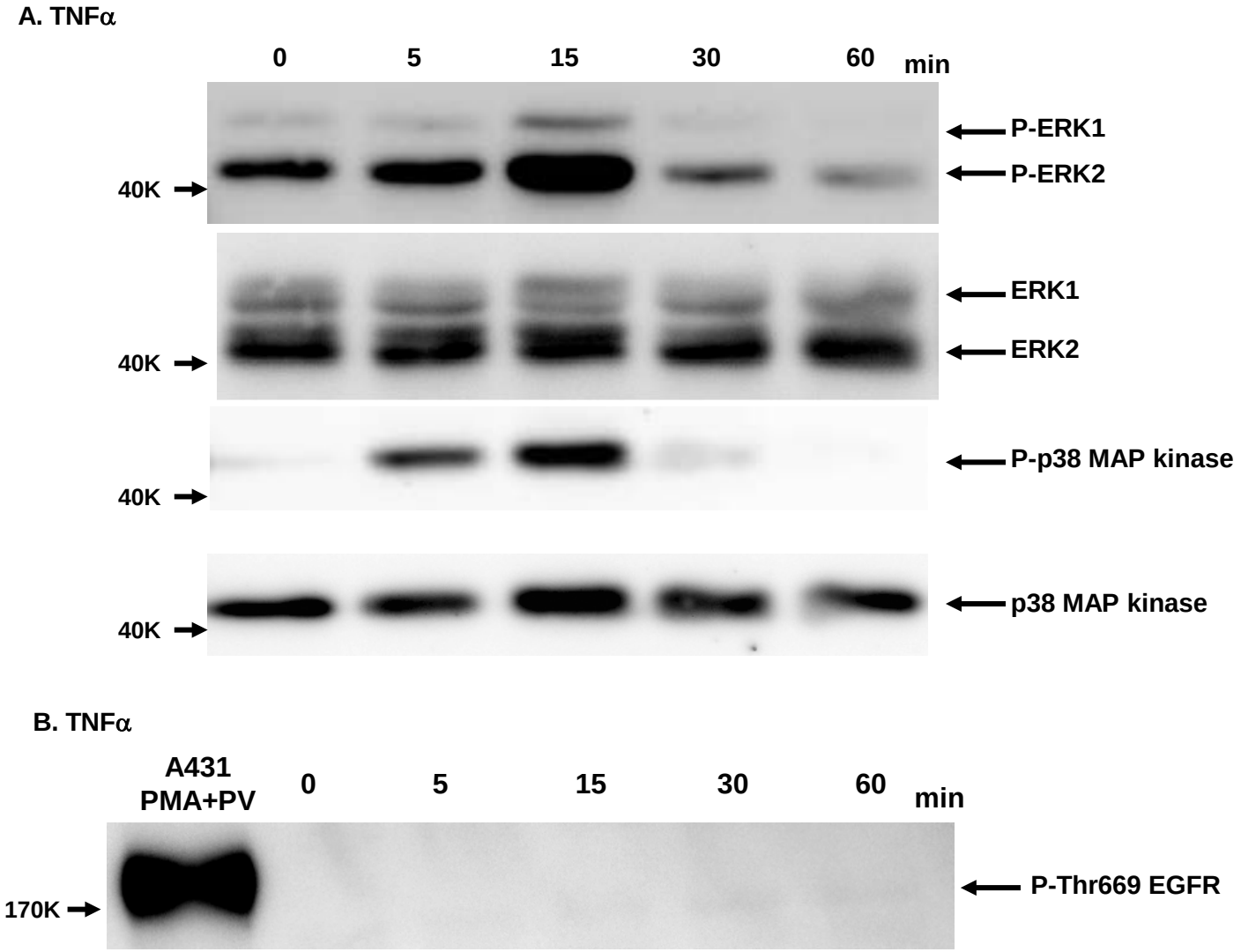

A. TNFα
0
5
15
30
60
min
P-ERK1
P-ERK2
40K
ERK1
ERK2
40K
P-p38 MAP kinase
40K
p38 MAP kinase
40K
B. TNFα
A431
PMA+PV
0
5
15
30
60
min
170K
P-Thr669 EGFR

Fig. 5

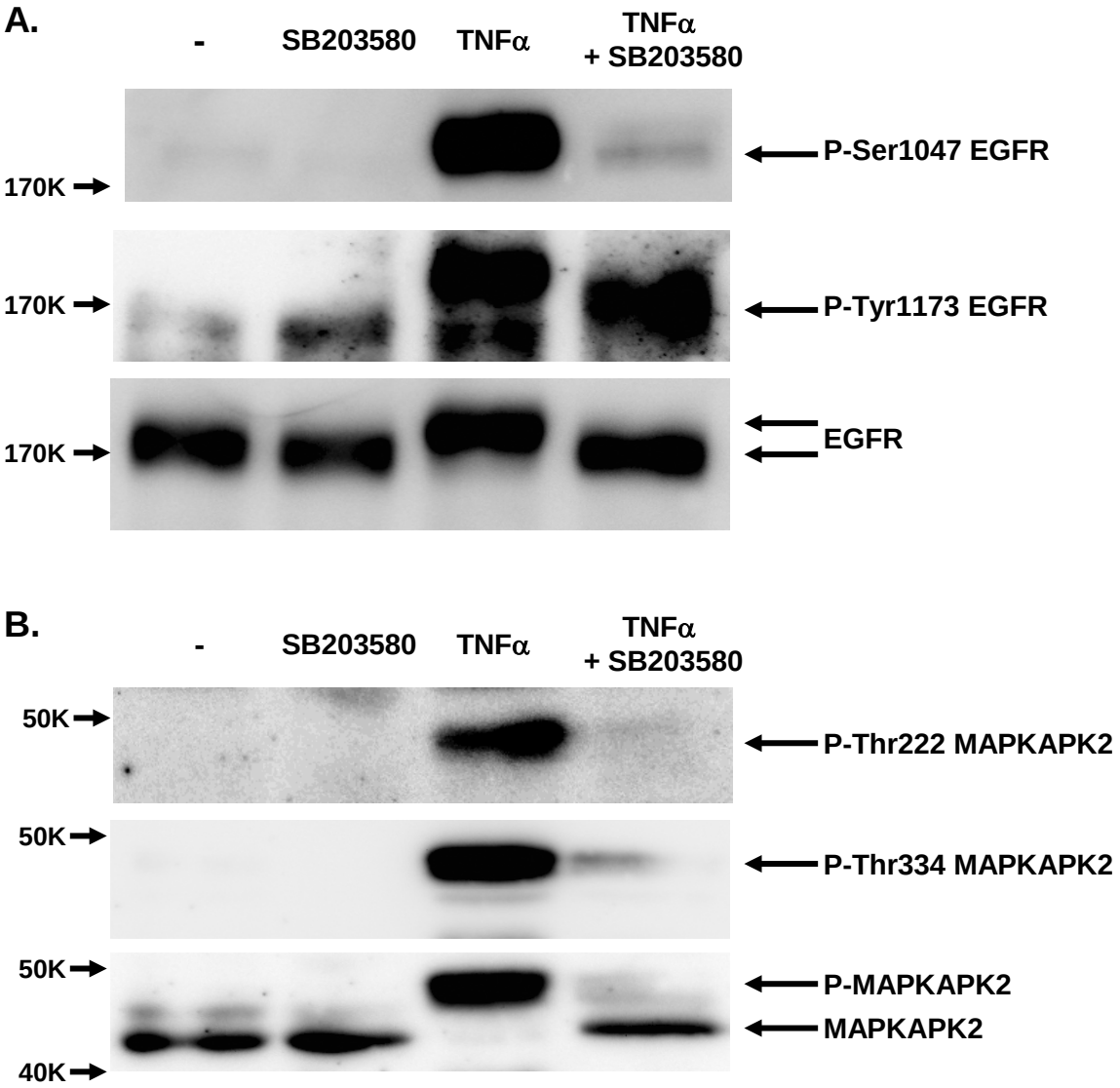

A.
-
SB203580
TNFα
TNFα
+ SB203580
170K
P-Ser1047 EGFR
170K
P-Tyr1173 EGFR
170K
EGFR
B.
-
SB203580
TNFα
TNFα
+ SB203580
50K
P-Thr222 MAPKAPK2
50K
P-Thr334 MAPKAPK2
50K
P-MAPKAPK2
MAPKAPK2
40K

Fig. 6

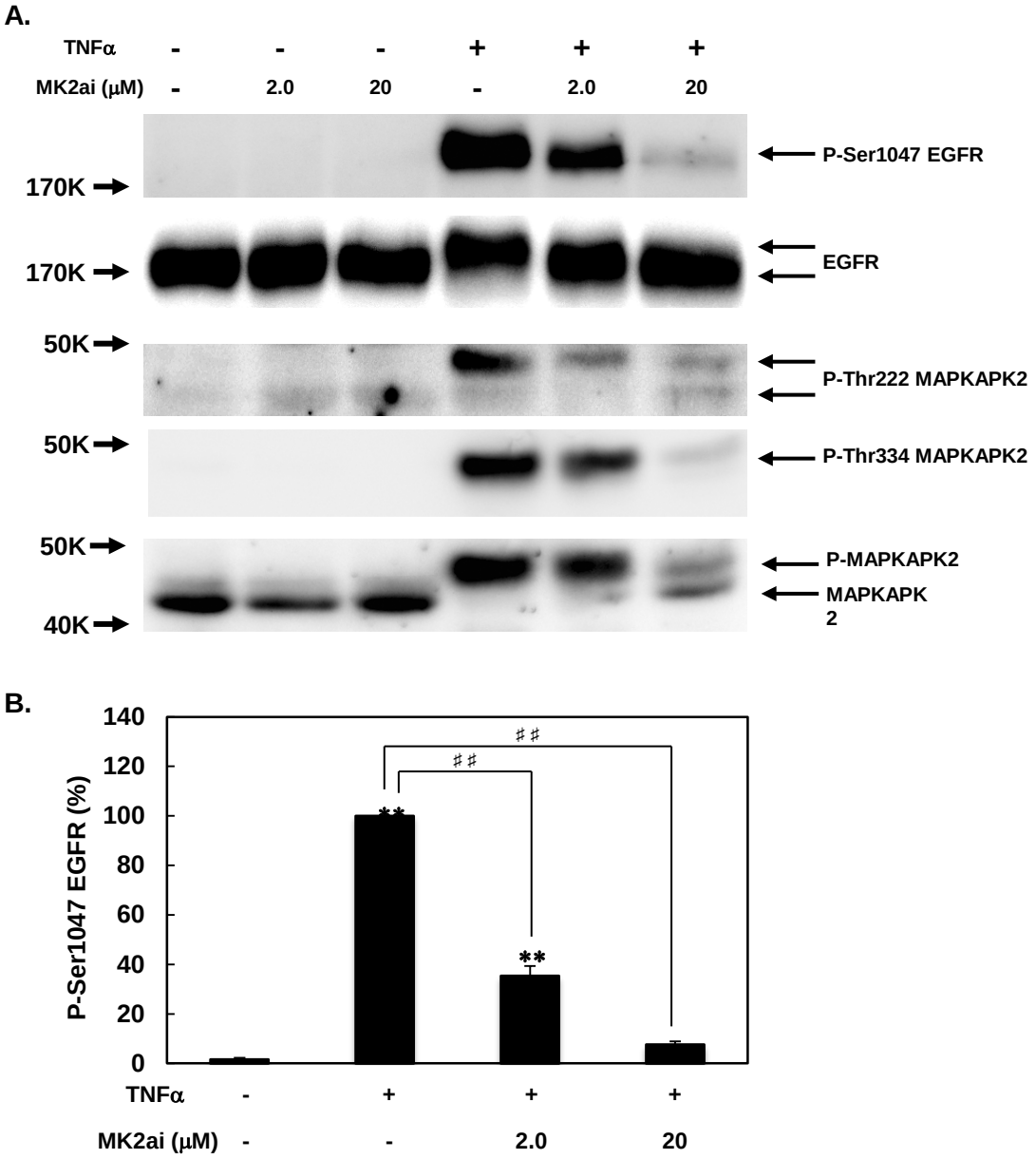

A.
TNFα - - - + + +
MK2ai (μM) - 2.0 20 - 2.0 20
170K
P-Ser1047 EGFR
170K
EGFR
50K
P-Thr222 MAPKAPK2
50K
P-Thr334 MAPKAPK2
50K
40K
P-MAPKAPK2
MAPKAPK 2
B.
P-Ser1047 EGFR (%)
140
120
100
80
60
40
20
0
♯♯
♯♯
**
**
TNFα - + + +
MK2ai (μM) - - 2.0 20

Fig. 7

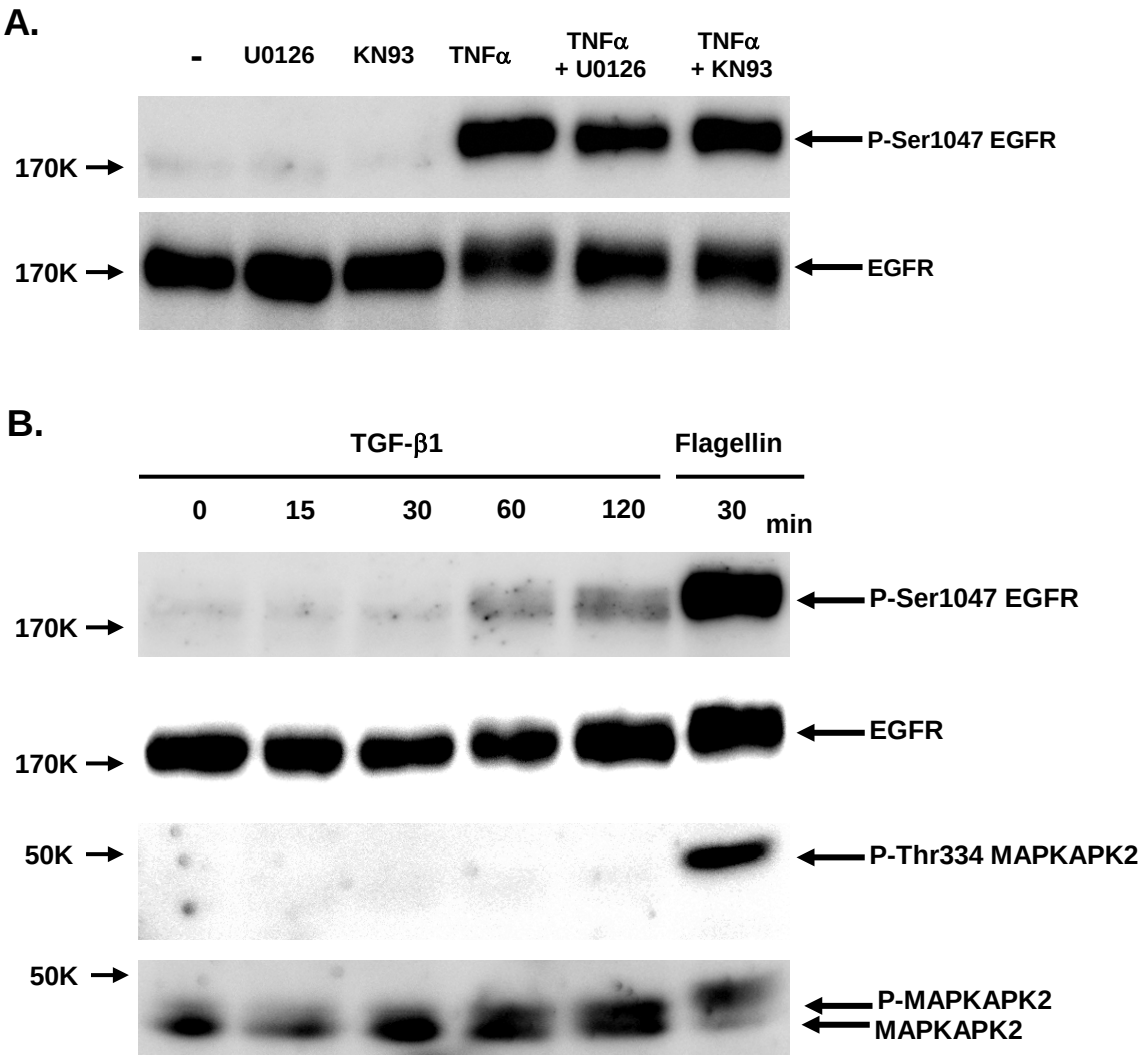
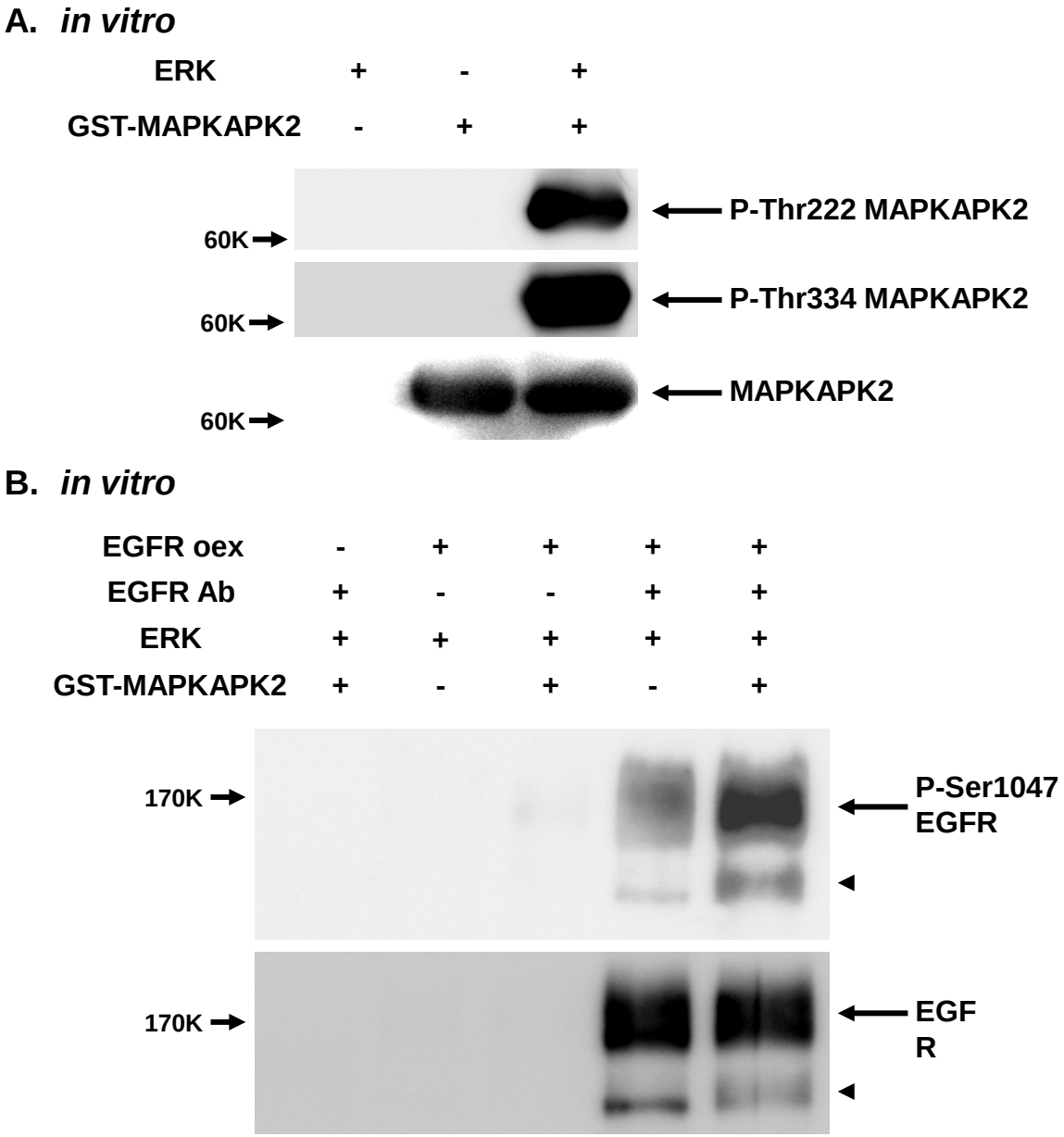

A.
-
U0126
KN93
TNFα
TNFα + U0126
TNFα + KN93
170K
P-Ser1047 EGFR
170K
EGFR
B.
TGF-β1
Flagellin
0
15
30
60
120
30
min
170K
P-Ser1047 EGFR
170K
EGFR
50K
P-Thr334 MAPKAPK2
50K
P-MAPKAPK2
MAPKAPK2

Fig. 8


A. in vitro
ERK + - +
GST-MAPKAPK2 - + +
60K
P-Thr222 MAPKAPK2
60K
P-Thr334 MAPKAPK2
60K
MAPKAPK2
B. in vitro
EGFR oex - + + + +
EGFR Ab + - - + +
ERK + + + + +
GST-MAPKAPK2 + - + - +
170K
P-Ser1047 EGFR
170K
EGF R