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Cdc42-dependent Mediation of UV-induced p38 Activation by G Protein $\beta\gamma$ Subunits*

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The β and γ subunits of heterotrimeric GTP-binding proteins (G$\beta\gamma$) were found to bi-directionally regulate the UV-induced activation of p38 and c-Jun NH_2-terminal kinase, and the UV-induced activation of p38 was reported to enhance the resistance of normal keratinocytes to apoptosis. However, the signaling pathway downstream of G$\beta\gamma$ for this UV-induced p38 activation is not known. Thus, we examined the role of the Rho GTPase family in the regulation of UV-induced p38 activation by G$\beta\gamma$. We found that overexpression of G$\beta\gamma$ increased the UV-induced activation of Cdc42 and that overexpression of constitutively active V12 Cdc42 increased the UV-induced p38 activation. Transfection of dominant negative N17 Cdc42 or small interfering RNA for Cdc42 blocked UV-induced p38 activation mediated by G$\beta\gamma$ in COS-1 and HaCaT cells. UV-induced p38 activation by G$\beta\gamma$ was blocked by overexpression of dominant negative p21-activated kinase (PAK)-interacting exchange factor β (βPix), and wild type βPix stimulated the UV-induced p38 activation, which was blocked by N17 Cdc42. G$\beta\gamma$ increased the UV-induced activation of Ras, and the overexpression of V12 Ras increased UV-induced p38 activation, which was blocked by dominant negative βPix. UV-induced p38 activation was inhibited by N17 Ras and a farnesyltransferase inhibitor, manumycin A. G$\beta\gamma$ also increased the UV-induced phosphorylation of the epidermal growth factor receptor (EGFR), and the UV-induced p38 activation was blocked by an EGFR kinase inhibitor, AG1478. From these results, we conclude that G$\beta\gamma$ mediates UV-induced activation of p38 in a Cdc42-dependent way and that EGFR, Ras, and βPix act sequentially upstream of Cdc42 in COS-1 and HaCaT cells.

The heterotrimeric GTP-binding regulatory proteins, known as G proteins, are composed of α, β, and γ subunits and transduce extracellular signals into intracellular signals by coupling the receptors and effectors (1). When a signaling molecule such as a hormone, a neurotransmitter, or a prostanoid binds to a G protein-coupled receptor (GPCR),[1] the receptor undergoes a conformational change (2). Such a conformational change in a receptor leads to the activation of G protein by stimulating the replacement of the GDP with GTP on the α subunit of G protein (Gα), which induces the dissociation of Gα-GTP from its $\beta\gamma$ dimer of G protein (G$\beta\gamma$) (3). Both the dissociated Gα-GTP and G$\beta\gamma$ subunits elicit cellular responses by regulating numerous effectors, which include adenylate cyclases, phospholipases, phosphodiesterases, protein kinases, mitogen-activated protein kinases (MAPKs), and ion channels (4, 5). The G protein signaling is terminated by the hydrolysis of GTP on the Gα subunit into GDP by intrinsic GTPase, which results in the formation of a heterotrimeric inactive structure composed of GDP-bound Gα and G$\beta\gamma$. Various molecules belonging to regulators of the G protein-signaling (RGS) family control the activity of intrinsic GTPase (6).

UV irradiation induces various cellular responses, which are believed to result from the activation of diverse cellular signal transduction systems. One of the signal transduction systems activated by UV is the MAPK family, which is composed of at least four families that include extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), p38, and ERK5/BMK1. The MAPK family regulates various cellular responses such as proliferation, differentiation, and apoptosis, which are activated by a variety of extracellular signals like growth factors, GPCR agonists, and cellular stress such as UV irradiation, hydrogen peroxide, and protein synthesis inhibitors (7, 8). MAPK is phosphorylated by MAPK kinase (MAPKK), which is phosphorylated and activated by MAPKK kinase (MAPKKK). This activation cascade of the MAPK family is well conserved evolutionarily (9). However, there is as much diversity in the regulatory molecules upstream of MAPKKK as there is among regulatory signals (10). A variety of signaling molecules have been reported that mediate the UV-induced MAPK activation in mammalian cells, such as the growth factor receptors that are activated ligand-independently (11, 12), as well as protein tyrosine phosphatase (13) and Ras (14).

The mammalian Rho family is a member of the Ras-related

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[1] The abbreviations used are: GPCR, G protein coupled receptor; βARKct, carboxyl-terminal region of β-adrenergic receptor kinase; βPix, PAK-interacting exchange factor β; DHm, dominant negative mutant of βPix; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PAK, p21-activated kinase; RTK, receptor tyrosine kinase; siRNA, small interfering RNA.

small GTPases and encompasses six classes, namely Rho, Rac, Cdc42, Rnd, RhoD, and TTF (15). The GTP-bound Rho proteins activate specific effectors, including serine/threonine kinases such as Rho-associated coiled-coil forming protein kinase (ROCK) and p21-activated kinase (PAK), and function as the key components in cellular processes that control the organization of the actin cytoskeleton, activate kinase cascades, regulate gene expression, regulate membrane trafficking, promote growth transformation, and induce apoptosis (16). Rho, Rac, and Cdc42 have been reported to regulate JNK and the p38 MAP kinase cascades (17–19) and thereby regulate gene transcription in a more direct way than via their effects on adhesion complexes (20).

Many GPCRs have the potential to regulate Rho family members by altering their abilities to associate with their regulatory proteins. Several studies indicate that GPCRs, which functionally couple to the heterotrimeric G proteins Gq, G12, and G13, can activate Rho family members (21). These receptors respond to important physiological agonists, including neuropeptides, neurotransmitters, and chemokines. The potential ability of these receptors to activate Rho family members indicates that many of the physiological responses mediated by these receptors may involve the activation of Rho family members.

In a previous experiment, the G protein $\beta\gamma$ subunit was found to bi-directionally regulate the UV-induced activations of p38 and JNK (22), suggesting that G proteins might mediate the MAPK activation stimulated by stresses like UV and by GPCR agonists. Such activation of p38 was found to enhance the resistance of normal human keratinocytes to apoptosis (23), but the signaling pathway downstream of $G\beta\gamma$ for the UV-induced activation of p38 is unknown. Because Rho family proteins play a role upstream of the p38 MAPK cascade, Rho family proteins are suggested as playing important roles in the activation of JNK and p38 induced by UV. However, it is not clear which Rho family protein is involved in the activation of p38 induced by stresses such as UV. Therefore, we undertook this study to determine which Rho protein is involved in the UV-induced activation of p38 mediated by $G\beta\gamma$ and which signaling molecules act upstream of Rho proteins. It was found that $G\beta\gamma$ mediates UV-induced activation of p38 in a Cdc42-dependant way and that epidermal growth factor receptor (EGFR), Ras, and the PAK-interacting exchange factor β (βPix) act sequentially upstream of Cdc42 in COS-1 and HaCaT cells.

EXPERIMENTAL PROCEDURES

Expression Plasmids—The expression plasmids for the β_1 and γ_2 isoforms of the G protein were subcloned into pcDNA3 expression vectors (Invitrogen) as described previously (22). A dominant negative mutant of PAK-interacting exchange factor β (βPix), βPix DHm (L238R,L239S), was constructed by replacing leucine 238 and leucine 239 with arginine and serine, respectively, in the Dbl-homology domain to remove the activity of the guanine nucleotide exchange factor (GEF) for Rac1 *in vivo* (24). The constitutively active and dominant negative mutants of Ras (V12 Ras and N17 Ras), Cdc42 (V12 Cdc42 and N17 Cdc42), and Rac1 (V12 Rac1 and N17 Rac1) were kindly provided by Dr. Chang-Dae Bae (Sungkyunkwan University, Korea). The FLAG-tagged wild type of p38 was gift form Dr. Dongeun Park (Seoul National University). The cDNA coding for the Ras binding domain of c-Raf1 (amino acids 1–149) fused to glutathione *S*-transferase and the Cdc42 and Rac binding domain of PAK (amino acids 67–150) fused to glutathione S-transferase were generously supplied by Dr. Walter Kolch (University of Glasgow, UK) and Dr. Jung-Won Lee (Seoul National University, Cancer Research Institute), respectively.

Cell Cultures and Transfections—African green monkey kidney cells, COS-1 (American Type Culture Collection), and HaCaT human keratinocytes were grown in Dulbecco's modified minimal essential media containing 10% fetal bovine serum, 100 IU/ml penicillin, and 50 μg/ml streptomycin and incubated in a CO_2 incubator at 37 °C. The COS-1 cells were split in 10-cm dishes 1 day prior to transfection, and the subconfluent cells were co-transfected with 5 μg of $G\beta_1$ and $G\gamma_2$ cDNA, together with additional DNA when necessary, using the DEAE-dextran method (25). The control cells were transfected with reagents without DNA or with the vector DNA without the insert. Twenty-four hours after transfection, the cells were re-plated in new dishes, and, 48 h later, the transfected COS-1 cells were rinsed twice with phosphate-buffered saline and irradiated with UVC (254 nm, 100 J/m^2). The UV-irradiated cells were harvested after incubation for a further 40 min.

The HaCaT cells were trypsinized and suspended in 400 μl of serum-free Dulbecco's modified Eagle's medium, and 5–10 μg of plasmid DNA or small interfereing RNA (siRNA) were added to cells. After the mixture was transferred to a 4-mm gap electroporation cuvette, electroporation was done using a Gene Pulser (Bio-Rad) at 200 V with 1.5 msec of pulse length and 1.5 s of pulse interval. Then the cells were diluted in growth medium and seeded onto culture plates. The cells were irradiated with UV 48 h after transfection.

Double-stranded RNA-mediated Interference—A double-stranded siRNA with 19-nucleotide duplex RNA and 2-nucleotide 3′ dTdT overhangs (sense, 5′-CUAUGCAGUCACAGUUAUGdTdT-3′; antisense, 5′-CAUAACUGUGACUGCAUAGdTdT-3′) targeting the sequence at the positions 115–135 relative to the start code (5′-AACTATGCATGCACAGTTATG-3′) of human Cdc42 was designed and purchased from Dharmacon Inc. The siRNA for luciferase GL2 (targeting sequence 5′-CGTACGCGGAATACTTCGA-3′) was used as a negative control. The siRNA was transfected by using electroporation method, and experiments were performed 48 h after transfection.

Immunoblot Analysis—UV-irradiated cells were harvested with a cell scraper in a lysis buffer containing 25 mM HEPES, pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, a protease inhibitor mixture (Roche Molecular Biochemicals), and 1% Triton X-100. The cells were lysed by incubating the suspension on ice for 30 min. The protein concentration of the lysate was measured using the bicinchoninic acid method. Fifty micrograms of the lysate protein was boiled in Laemmli buffer, separated on a 10 or 15% SDS-polyacrylamide gel, and then transferred to a nitrocellulose membrane. The blot was blocked with 5% nonfat milk in TTBS (20 mM Tris-Cl, pH 7.5, 500 mM NaCl, and 0.1% Tween 20) for 1 h, and then incubated in a cold room overnight with a specific antibody. The antibody against Gβ (SW) was prepared as described previously (22), antibodies against p38 and EGFR were purchased from Santa Cruz Biotechnology, antibodies against phosphorylated p38 (Thr-180/Tyr-182), phosphorylated EGFR (Tyr1068), and Myc were from Cell Signaling Technology, antibodies against Cdc42 and Rac1 were from Signal Transduction, the FLAG antibody was from Sigma, and the Ras antibody came from Upstate Biotechnology. The nitrocellulose membrane was subsequently washed with TTBS and incubated with a peroxidase-labeled goat anti-rabbit IgG antibody (1:2000 dilution, Pierce) for 2 h at room temperature. The blot was washed with TTBS and incubated with an enhanced chemiluminescence substrate mixture (Pierce). The blot was then exposed on x-ray film (AGFA Curix RPI) to obtain an image. The density of the visualized band was quantified using an image analyzer (Bio-Rad Laboratories, model GS-700), and the relative band density was expressed as a multiple of the band density in the UV-irradiated vector-transfected cells.

Assay of p38 Activity—The activity of p38 was measured by Western blot analysis of the phosphorylation of either endogenous p38 or transfected FLAG-tagged p38 using a phosphorylated p38 specific antibody. For analysis of FLAG-tagged p38 phosphorylation, UV-irradiated cells were lysed in the lysis buffer, and then 500 μg of cell extract was incubated with 5 μg of anti-FLAG antibody (Sigma) in a cold room overnight and then with protein G-Sepharose-conjugated beads (Pierce) for 2 h. The immune complex was collected by centrifugation and washed four times with lysis buffer. The proteins were resolved in 10% SDS-PAGE and transferred to nitrocellulose. Immunoblot analysis was performed using anti-phospho-p38 and anti-FLAG antibodies.

Assay of Ras, Rac1, and Cdc42 activity—Ras activity was assayed by analyzing the binding of the activated Ras to the Ras binding domain of Raf-1 (26). Briefly, after the endogenous Ras was activated by UV irradiation, the cells were lysed in a lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture). The cell lysate (200 μg) was incubated with glutathione-Sepharose coupled to the peptides corresponding to the Ras binding domain of Raf-1 for 1 h at 4 °C. The beads were precipitated by centrifugation and washed three times with the lysis buffer. The bound proteins were eluted in a Laemmli sample treatment buffer, heated for 5 min, and then separated on 15% SDS-PAGE. Proteins in the gel were transferred to a

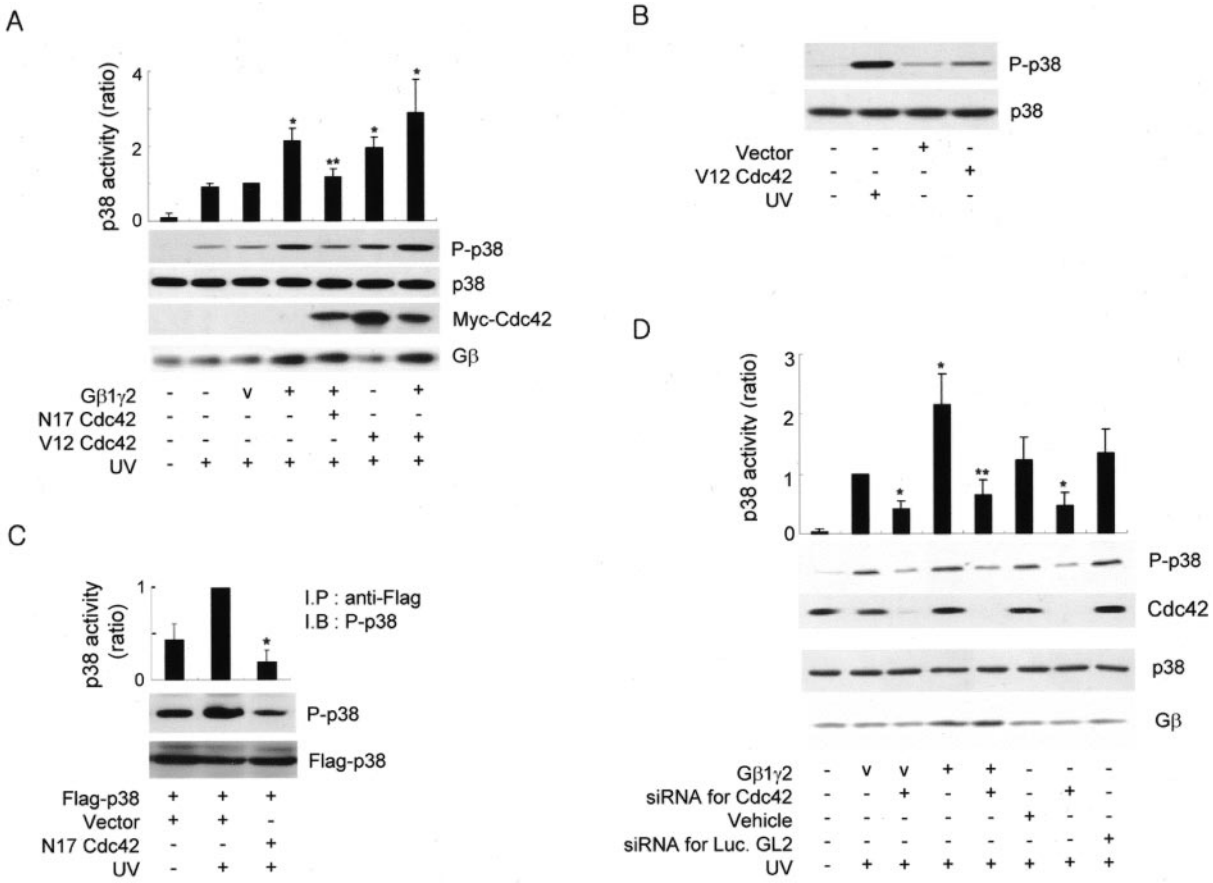

FIG. 1. **Involvement of Cdc42 in the UV-induced activation of p38 mediated by Gβγ.** *A*, effects of Cdc42 overexpression on the UV-induced activation of p38 mediated by Gβγ. *B*, activation of p38 by constitutively active V12 Cdc42 in the absence of UV stimulation. *C*, inhibition of UV-induced p38 activation by dominant negative N17 Cdc42 as assessed by FLAG-p38 phosphorylation assay. *D*, effects of overexpression of siRNA for Cdc42 on the UV-induced activation of p38 mediated by Gβγ. COS-1 cells were respectively transfected with the constitutively active or dominant negative forms of Cdc42 (V12 Cdc42 and N17 Cdc42) with $G\beta_1\gamma_2$ using a DEAE-dextran method (*A* and *B*). The siRNAs for Cdc42 and luciferase GL2 (*Luc. GL2*) were transfected by electroporation into HaCaT cells (*D*). After 72 h (48 h for HaCaT cells), the cells were exposed to UV irradiation (100 J/m^2), incubated for a further 40 min, harvested, and lysed. The lysate was separated by 15% SDS-PAGE, and the proteins were transferred to nitrocellulose paper. The blot was incubated with antibodies directed against Gβ, p38, phosphorylated p38, and Myc and then with a peroxidase-labeled goat anti-rabbit IgG antibody. Proteins were visualized by incubating the blot with an enhanced chemiluminescence substrate mixture and by exposure to x-ray film. The density of the phosphorylated protein band was measured with a densitometer, and the p38 activity was expressed as a ratio of the band density to that of vector-transfected COS-1 cells. Other COS-1 cells were co-transfected with N17 Cdc42 with FLAG-tagged p38. After 72 h, the transfected cells were exposed to UV irradiation (100 J/m^2), and the activity of p38 was determined by immunoprecipitation (*IP*) with the FLAG antibody and by Western blot (*IB*) using an antibody specific to phosphorylated p38 (*C*). The blots shown are representative of at least 3–5 independent experiments, and the histograms represent the average and standard deviations. The asterisk (*) represents a statistically significant difference from the vector-transfected control (*V*), and ** represents a statistically significant difference from the Gβγ-transfected cells ($p < 0.05$, Mann-Whitney *U* test).

nitrocellulose membrane, and immunoblot analysis was performed using an anti-Ras antibody (Upstate Biotechnology). The activities of Cdc42 and Rac1 were assayed by analyzing the binding of the activated proteins to the Cdc42 and Rac binding domain of PAK (amino acid residues 67–150) fused to glutathione *S*-transferase. Bound Cdc42 and Rac1 were detected by Western blotting using an anti-Cdc42 and a Rac1 antibody, respectively (27).

Data Analysis—At least three independent experiments were conducted. The non-parametric Mann-Whitney *U* test was used to analyze the averaged values, and a p value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

This study aimed to determine which Rho family protein is involved in the UV-induced activation of p38 mediated by Gβγ and which signaling molecules act upstream of Rho proteins. The main finding of this study was that Cdc42, and not Rac1, is required in the UV-induced activation of p38 mediated by Gβγ in COS-1 and HaCaT cells and that EGFR, Ras, and βPix act sequentially upstream of Cdc42.

Cdc42, Not Rac1, Is Involved in the UV-induced Activation of p38 Mediated by Gβγ—The G protein βγ subunit was found to mediate the UV-induced activation of p38 in a previous study (22), but the signaling pathways connecting Gβγ and p38 activation were not elucidated. Rho family proteins are involved in the activation of p38 by various stimuli, including cellular stresses; however, it is not clear whether the Rho protein is involved in the UV-induced activation of p38 mediated by Gβγ. Consequently, this study was undertaken to study the role of Rho proteins in the UV-induced activation of p38 mediated by Gβγ, and, thus, the effect of the overexpression of mutant Cdc42, Rac1, or RhoA on the UV-induced activation of p38 was examined. The overexpression of the constitutively active V12 Cdc42 increased UV-induced p38 activation to a level comparable with that achieved by Gβγ overexpression, which was about twice that of the vector-transfected control. The co-expression of V12 Cdc42 and Gβγ slightly augmented the UV-induced p38 activation caused by the expression of either V12 Cdc42 or Gβγ alone, but without statistical significance (Fig. 1*A*). Expression of V12 Cdc42 alone without UV stimulation increased p38 activity to ~40% of that achieved by UV stimulation (Fig. 1*B*). Moreover, the overexpression of dominant

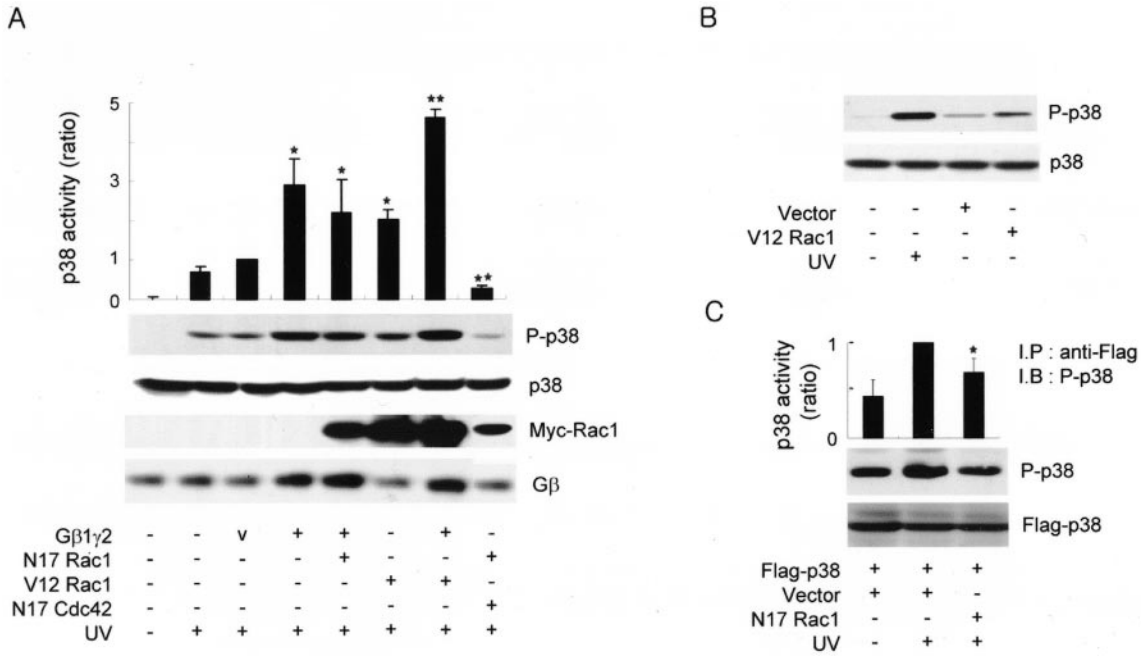

FIG. 2. **Mediation of UV-induced p38 activation in a Rac1-independent manner by Gβγ.** *A*, effects of Rac1 overexpression on the UV-induced activation of p38 mediated by Gβγ. *B*, activation of p38 by constitutively active V12 Rac1 in the absence of UV stimulation. *C*, inhibition of UV-induced p38 activation by dominant negative N17 Rac1 assessed by FLAG-p38 phosphorylation assay. COS-1 cells were transfected, respectively, with the constitutively active or dominant negative forms of Rac1 (V12 Rac1 or N17 Rac1) and $G\beta_1\gamma_2$ using a DEAE-dextran method (*A* and *B*). After 72 h, the cells were exposed to UV irradiation (100 J/m^2), and the activity of p38 was analyzed by Western blot using an antibody specific to phosphorylated p38. Other COS-1 cells were co-transfected with N17 Rac1with FLAG-tagged p38, and, after 72 h, the transfected cells were exposed to UV irradiation (100 J/m^2). The activity of p38 was determined by immunoprecipitation (*IP*) with FLAG antibody and by Western blot (*IB*) using an antibody specific to phosphorylated p38 (*C*). The p38 activity was expressed as a ratio of the band density to that of vector-transfected cells. The blots shown are representative of at least 3–5 independent experiments, and the histograms represent the average and standard deviations. The asterisk (*) represents a statistically significant difference from the vector-transfected control (*V*), and ** represents a statistically significant difference from the Gβγ-transfected cells ($p < 0.05$, Mann-Whitney *U* test).

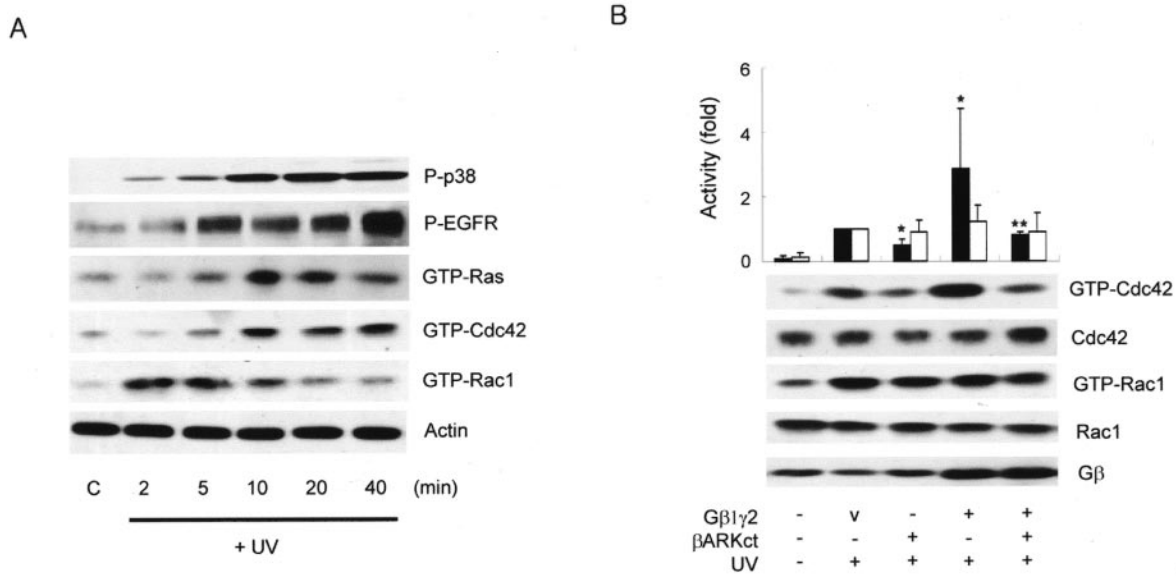

FIG. 3. **Effects of Gβγ on the UV-induced activation of Cdc42 and Rac1.** *A*, time course of EGFR, Ras, Cdc42, Rac1, and p38 activation following UV irradiation. *B*, effects of Gβγ on the UV-induced activation of Cdc42 and Rac1. For the time course analysis, COS-1 cells were irradiated with UV, and then analysis was performed at the indicated times. The phosphorylation of EGFR and p38 was detected by Western blot using an antibody specific to phosphorylated EGFR or p38. The activities of Cdc42 and Rac1 were assessed by analyzing their respective binding to the Cdc42/Rac1 binding domain of PAK1 protein, and the activity of Ras was assessed by measuring the binding to a Raf domain. Other COS-1 cells were transfected with $G\beta_1\gamma_2$ and/or βARK by the DEAE-dextran method; at 72 h after transfection, the cells were irradiated with UV, and then Cdc42 and Rac1 activities were assayed 40 and 2 min later, respectively. The blots are representative of 3–5 independent experiments, and the histograms represent their average and standard deviations. The *filled bars* represent Cdc42 activity, and the *empty bars* represent Rac1 activity (*B*). The asterisk (*) represents a statistically significant difference from the vector-transfected control (*V*), and ** represents a statistically significant difference from the Gβγ-transfected cells ($p < 0.05$, Mann-Whitney *U* test).

negative N17 Cdc42 almost completely blocked the increase in the UV-induced p38 activation caused by Gβγ overexpression (Fig. 1*A*). Because the average transfection efficiency of DEAE-dextran method was ~30%, the UV-induced phosphorylation of the co-transfected FLAG-tagged p38 was analyzed to control the transfection efficiency. The overexpression of N17 Cdc42

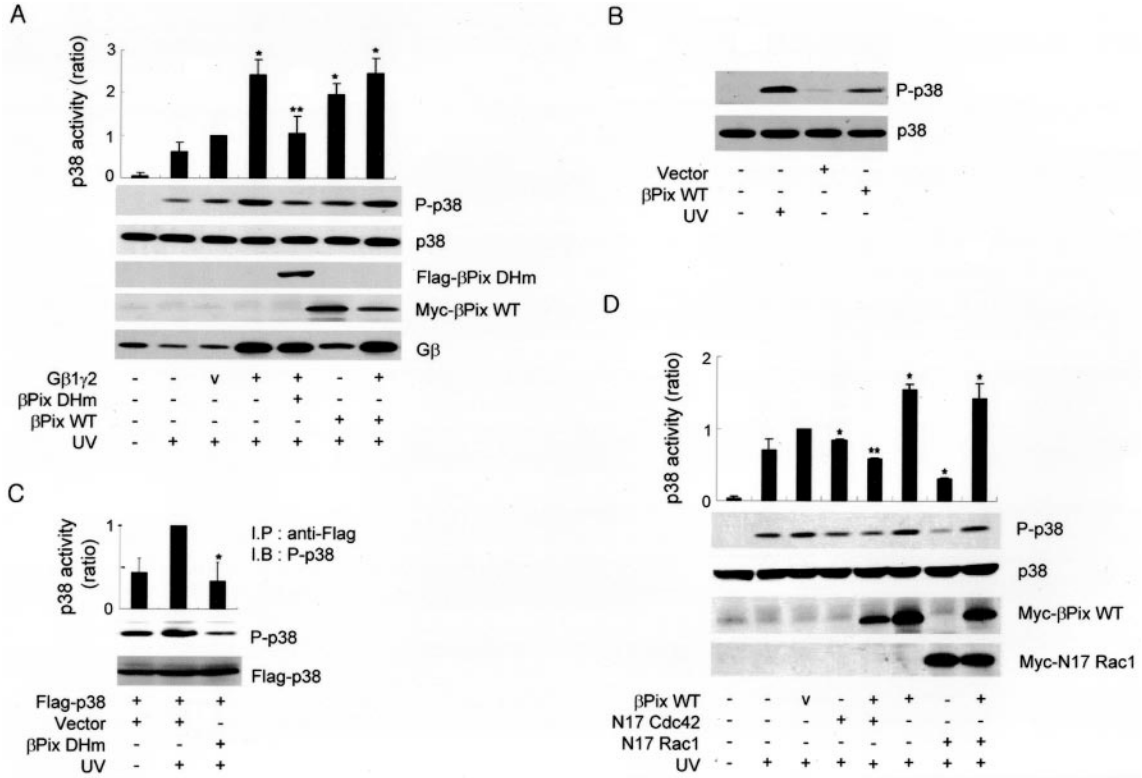

FIG. 4. **Involvement of βPix upstream of Cdc42 in the UV-induced activation of p38 mediated by Gβγ in COS-1 cells.** *A*, effects of βPix overexpression on the UV-induced activation of p38 mediated by Gβγ. *B*, activation of p38 by wild type βPix in the absence of UV stimulation. *C*, inhibition of UV-induced p38 activation by dominant negative βPix DHm assessed by a FLAG-p38 phosphorylation assay. *D*, inhibition of the βPix-mediated UV-induced activation of p38 by dominant negative N17 Cdc42. The COS-1 cells were transfected with either the wild type (*WT*) or dominant negative (*DHm*) βPix with $G\beta_1\gamma_2$ cDNA by the DEAE-dextran method (*A* and *B*). Other COS-1 cells were co-transfected with wild type βPix and the dominant negative N17 Cdc42 or N17 Rac1 (*D*). After 72 h, the cells were exposed to UV irradiation (100 J/m^2), and the activity of p38 was analyzed by Western blot using an antibody specific to phosphorylated p38. COS-1 cells were also co-transfected with βPix DHm with FLAG-tagged p38. After 72 h, the transfected cells were exposed to UV irradiation (100 J/m^2), and the activity of p38 was determined by immunoprecipitation (*IP*) with a FLAG antibody and by Western blot (*IB*) using an antibody specific to phosphorylated p38 (*C*). The blots shown are representative of at least 3–5 independent experiments, and the histograms represent the average and standard deviations. The asterisk (*) represents a statistically significant difference with respect to the vector-transfected control (*V*), and ** represents a statistically significant difference with respect to Gβγ-transfected cells (*A*) or to the wild type βPix-transfected cells (*D*) ($p < 0.05$, Mann-Whitney *U* test).

decreased UV-induced p38 activation to 21% of the control in the absence of Gβγ overexpression when the phosphorylation of co-transfected FLAG-tagged p38 was analyzed (Fig. 1*C*). In addition, the transfection of siRNA for Cdc42 reduced UV-induced p38 activation to 30% in the presence of Gβγ overexpression and 41% in the absence of Gβγ overexpression. Transfection of siRNA for luciferase GL2, an unrelated siRNA, did not change the UV-induced p38 activity (Fig. 1*D*). All of these results demonstrate that Cdc42 is involved in the UV-induced p38 activation mediated by Gβγ and that Gβγ mediates the UV-induced p38 activation via a Cdc42-dependent pathway. This is the first report, to our knowledge, which shows that Cdc42 is involved in UV-induced p38 activation, although Rac1 has been reported to mediate UV-induced p38 activation, resulting in the apoptosis of Rat-2 fibroblasts (28).

Alternatively, the overexpression of constitutively active V12 Rac1 increased the UV-induced p38 activation to a level comparable with that achieved by Gβγ overexpression. It also further and significantly increased the p38 activity caused by Gβγ overexpression from 3.1 to 4.6 times that of the control ($p = 0.003$), displaying an additive relation between V12 Rac1 and Gβγ in COS-1 cells (Fig. 2*A*). The expression of V12 Rac1 alone without UV stimulation increased p38 activity to ~35% of that achieved by UV-stimulation (Fig. 2*B*). The overexpression of dominant negative N17 Rac1 reduced the UV-induced p38 activation to 69% of the vector-transfected control in the absence of Gβγ overexpression in an analysis of FLAG-tagged p38 phosphorylation in COS-1 cells (Fig. 2*C*). However, the simultaneous overexpression of N17 Cdc42 and N17 Rac1 blocked UV-induced p38 activation almost completely (Fig 2*A*). This result indicates that Rac1 might mediate the UV-induced activation of p38 in a Gβγ-independent way and suggests that Rac1 and Gβγ/Cdc42 might mediate UV-induced p38 activation through independent and separate pathways. The overexpression of the constitutively active or dominant negative mutant of RhoA protein did not induce significant changes in UV-induced p38 activation in the absence or in the presence of Gβγ overexpression in COS-1 cells (data not shown).

To assess the respective roles of Cdc42 and Rac1 in UV-induced p38 activation, the time courses of their activations and the effects of Gβγ overexpression on their activations were analyzed by measuring their binding to the Cdc42/Rac binding domain of PAK. p38 activity started to increase 2 min after UV irradiation and then continued to increase to reach a peak at 40 min. However, Cdc42 activity started to increase at 5 min and continued to increase until 40 min after UV irradiation (Fig. 3*A*). The overexpression of Gβγ resulted in a 2.8-fold increase in Cdc42 activity 40 min after UV irradiation, which was blocked by the overexpression of Gβγ-sequestering βARKct (carboxyl-terminal region of β-adrenergic receptor kinase) (Fig. 3*B*). On the other hand, Rac1 showed peak activity at 2 min after UV irradiation and then decreased to remain slightly above the basal level after 10 min (Fig. 3*A*). The overexpression of Gβγ and βARK did not significantly change Rac1 activity 2

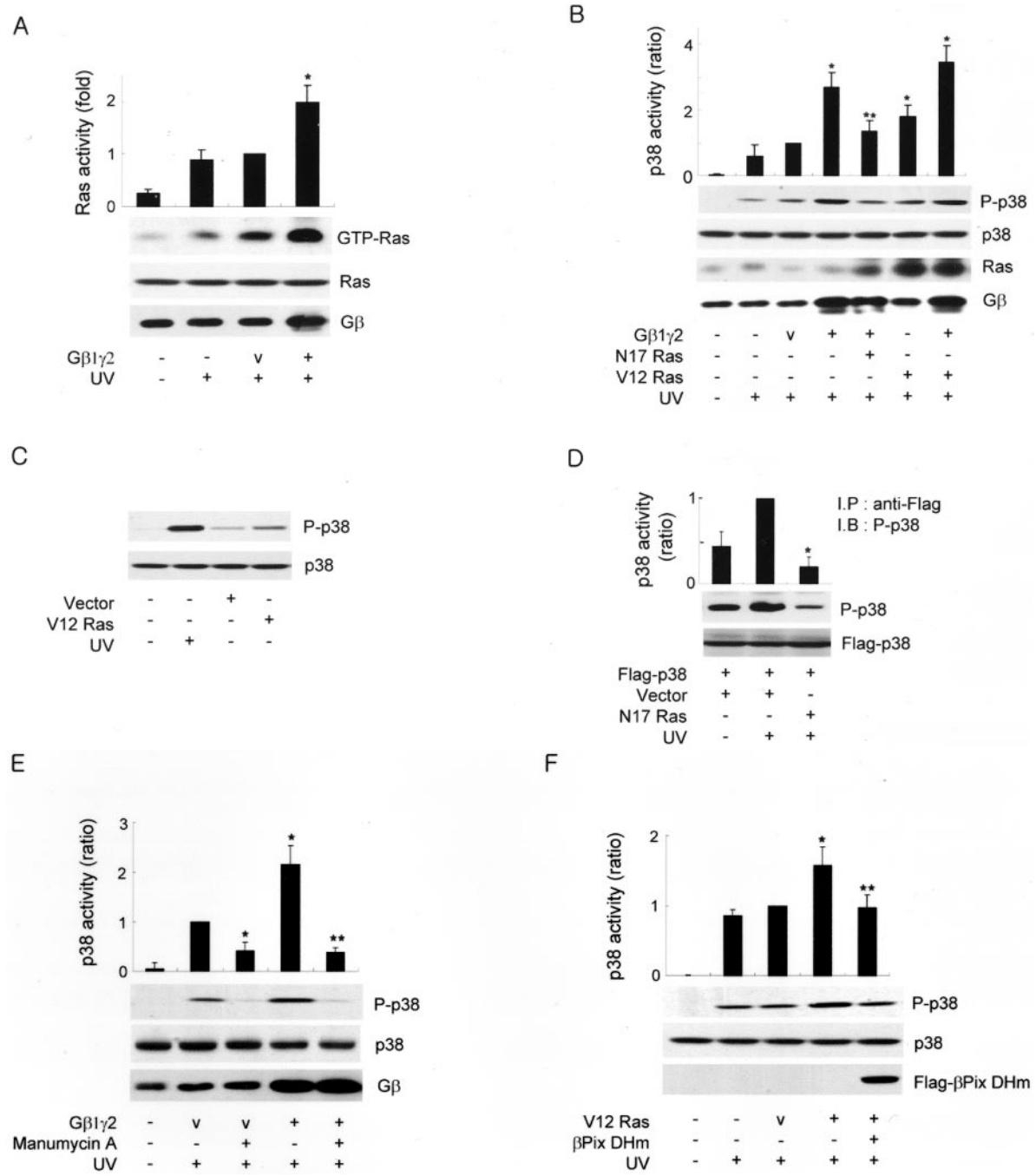

FIG. 5. **Involvement of Ras in the UV-induced activation of p38 mediated by Gβγ in COS-1 cells.** *A*, increase in the UV-induced activation of Ras by Gβγ overexpression. *B*, effects of Ras overexpression on the UV-induced activation of p38 mediated by Gβγ. *C*, activation of p38 by constitutively active V12 Ras in the absence of UV stimulation. *D*, inhibition of UV-induced p38 activation by dominant negative N17 Ras assessed by a FLAG-p38 phosphorylation assay. *E*, inhibition of the UV-induced activation of p38 by manumycin A. *F*, inhibition of the Ras-mediated UV-induced activation of p38 by dominant negative βPix in COS-1 cells. COS-1 cells were transfected with $G\beta_1\gamma_2$, constitutively active Ras (V12 Ras), dominant negative Ras (N17 Ras), or βPix DHm by the DEAE-dextran method. After 72 h, the cells were exposed to UV irradiation (100 J/m^2), Ras activity was determined by measuring Ras binding to the Ras binding domain of Raf-1 at 40 min after UV irradiation (*A*), and the activity of p38 was analyzed by Western blot using an antibody specific to phosphorylated p38 (*B*, *C*, and *F*). p38 activity was also measured after pretreatment with 5 μM manumycin A (Calbiochem) for 3 h before UV irradiation (*E*). Other COS-1 cells were co-transfected with N17 Ras with FLAG-tagged p38. After 72 h, the transfected cells were exposed to UV irradiation (100 J/m^2), and the activity of p38 was determined by immunoprecipitation (*IP*) with a FLAG antibody and by Western blot (*IB*) using an antibody specific to phosphorylated p38 (*D*). The activities of Ras and p38 are expressed as ratios of the band density to that of the vector-transfected COS-1 cells. The blots shown are representative of at least 3–5 independent experiments, and the histograms represent the average and standard deviations. The asterisk (*) represents a statistically significant difference *versus* the vector-transfected control (*V*), and ** represents a statistically significant difference *versus* the Gβγ-transfected cells (*B* and *E*) or *versus* V12 Ras-transfected cells (*F*) ($p < 0.05$, Mann-Whitney *U* test).

min after UV irradiation (Fig. 3*B*). However, the UV-induced activation of p38 at 2 min was blocked almost completely by N17 Rac1 overexpression, but not by N17 Cdc42 (data not shown). This finding indicates that Rac1 might induce p38 activation immediately after UV irradiation in a Gβγ-independent manner, but Cdc42 might mediate the delayed and sustained p38 activation induced by UV irradiation in a Gβγ-dependent manner in COS-1 cells. This suggests that UV immediately activates p38 via Rac1 and then sustains p38 activity via Cdc42 and Rac1. It also implies that Gβγ stimulates the

delayed and sustained UV-induced p38 activation through Cdc42 but that it is not involved in the UV-induced p38 immediate activation mediated by Rac1. Thus, it is speculated from these findings that UV irradiation activates G proteins by unknown mechanisms and that the activated Gβγ induces the prolonged activation of p38 via Cdc42 to induce cellular responses. However, further study is needed to determine whether p38 activation mediated by Cdc42 or Rac1 might play different roles in inducing cellular responses.

It has been reported that Gβγ activates MKK3 in a Rac- and Cdc42-dependent manner and activates MKK6 in a Rho-, Rac-, and Cdc42-dependent manner (29). Cdc42 was shown to activate both the p38 and JNK pathways, and Rac1 was shown to activate p38 (17, 18). In the present study, however, Gβγ was found to activate Cdc42 but not Rac1 in UV-induced p38 activation. A similar differential regulation of Cdc42 and Rac was found during FcR-mediated phagocytosis, where Vav was found to specifically regulate Rac activation, but not Cdc42 activation, and both Cdc42 and Rac were activated at the FcR-dependent phagosomes through distinct pathways (30). The molecular mechanism of such specificity for activation of the Rho family proteins is unclear, but we speculated that it might reflect the difference in cell types and stimulating signals.

βPix Acts Upstream of Cdc42 in the UV-induced Activation of p38 Mediated by Gβγ—To find the signaling molecules acting upstream of Cdc42 in the UV-induced p38 activation mediated by Gβγ, the involvement of βPix was examined. βPix is a recently identified guanine nucleotide exchange factor family for the Rho G proteins, Cdc42 and Rac, and it was reported to play an essential role in growth factor-stimulated p38 activation (24). However, it is not known whether βPix is involved in stress-induced p38 activation, and, thus, the effect of the overexpression of the wild type and dominant negative βPix on UV-induced p38 activation was analyzed. Overexpression of the wild type βPix increased the UV-induced p38 activation by approximately twice that of the vector-transfected control. However, this did not result in a further augmentation of the UV-induced p38 activation caused by Gβγ overexpression (Fig. 4*A*). The overexpression of wild type βPix alone also increased the p38 activity to 40% of that achieved by UV-stimulation (Fig. 4*B*). In contrast, the dominant negative mutant, βPix DHm, blocked most of the increased UV-induced p38 activity that resulted from the overexpression of $G\beta_1\gamma_2$ (Fig. 4*A*), and overexpression of βPix DHm blocked p38 activation to 31% of that of the vector-transfected control in a FLAG-tagged p38 phosphorylation assay (Fig. 4*C*). These results suggest that βPix is involved in the signaling pathway of the UV-induced p38 activation mediated by both exogenous and endogenous Gβγ in COS-1 cells and that Gβγ mediates the UV-induced p38 activation in a βPix-dependent manner.

To determine whether βPix acts upstream of Cdc42 alone and not upstream of Rac1 in the UV-induced p38 activation mediated by Gβγ, the effect of the simultaneous overexpression of wild type βPix and the dominant negative mutant of Cdc42 or Rac1 was analyzed. The overexpression of the dominant negative N17 Cdc42 blocked the increase in UV-induced p38 activation caused by the overexpression of wild type βPix (Fig. 4*D*). Furthermore, the overexpression of βPix increased Cdc42 activity 40 min after UV irradiation (data not shown). However, N17 Rac1 did not block the increase in UV-induced p38 activation caused by the overexpression of wild type βPix (Fig. 4*D*), indicating that βPix might regulate UV-induced p38 activation in a Rac1-independent pathway. This result indicates that βPix causes UV-induced p38 activation via a Cdc42-dependent pathway and that βPix acts upstream of Cdc42 in the UV-induced p38 activation pathway mediated by Gβγ. These findings are in agreement with the finding that both βPix and Cdc42 are involved in the UV-induced p38 activation mediated by Gβγ, but that Rac1 is not involved.

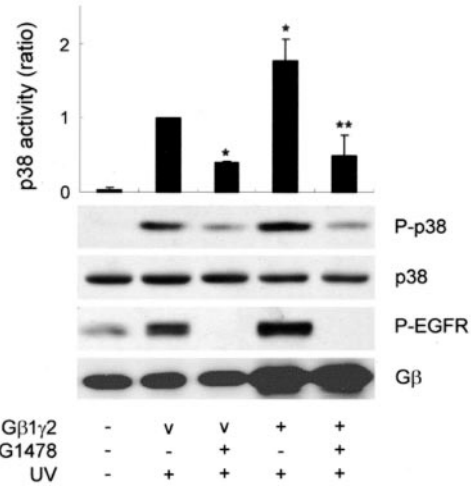

FIG. 6. **Involvement of the EGFR phosphorylation in the UV-induced activation of p38 mediated by Gβγ in COS-1 cells.** COS-1 cells were transfected with $G\beta_1\gamma_2$ by the DEAE-dextran method, and 72 h after transfection the cells were exposed to UV irradiation (100 J/m^2) with or without pretreatment with 20 μM AG1478 (Calbiochem) for 30 min. Then, the EGFR phosphorylation was assessed at 40 min after UV irradiation by Western blot using an antibody specific to phosphorylated EGFR. The blots are representative of 3–5 independent experiments, and the histograms represent their averages and standard deviations. The asterisk (*) represents a statistically significant difference *versus* the vector-transfected control (*V*), and ** represents a statistically significant difference *versus* the Gβγ-transfected cells ($p < 0.05$, Mann-Whitney *U* test).

Because βPix is known to have guanine nucleotide exchange factor activity for both Cdc42 and Rac, they both could be activated by βPix during the UV-induced p38 activation mediated by Gβγ. However, only Cdc42 was found to be involved in this study, indicating the presence of a mechanism that enables βPix to activate Cdc42 selectively, but not Rac1. However, the extent and selectivity of the Pix isoforms required to activate Cdc42 and Rac are not clearly understood (31). Therefore, further study on the mechanism of the differential activations of Cdc42 and Rac by βPix would contribute to a better understanding of the selective activation of Cdc42 by Gβγ.

The above result shows that βPix can play a crucial role in the UV-induced p38 activation mediated by Gβγ. This finding, together with a recent report showing that βPix plays an essential role in both p38 activation and cytoskeleton rearrangement by platelet-derived growth factor (24), suggests that βPix might mediate cellular stress signals, as well as growth factor signals, to induce p38 activation, cytoskeleton rearrangements, neuronal degeneration, and carcinogenesis (32). Furthermore, various GPCR agonists have been reported to activate p38, and both Gα and Gβγ are involved in p38 activation (5). βPix was found to mediate p38 activation by Gβγ in this study. Consequently, we speculate that various GPCR agonists, such as hormones and neurotransmitters, can regulate βPix and p38 activity through Gβγ to induce various cellular responses, including a cytoskeleton rearrangement.

Ras Acts Upstream of βPix in the UV-induced Activation of p38 Mediated by Gβγ—To find a signaling molecule acting upstream of βPix, the role of Ras in the UV-induced activation of p38 mediated by Gβγ was analyzed. This was because Ras is activated by UV irradiation and regulates a variety of downstream signaling molecules, including p38 (33, 34). The effect of Gβγ overexpression on the UV-induced activation of Ras and

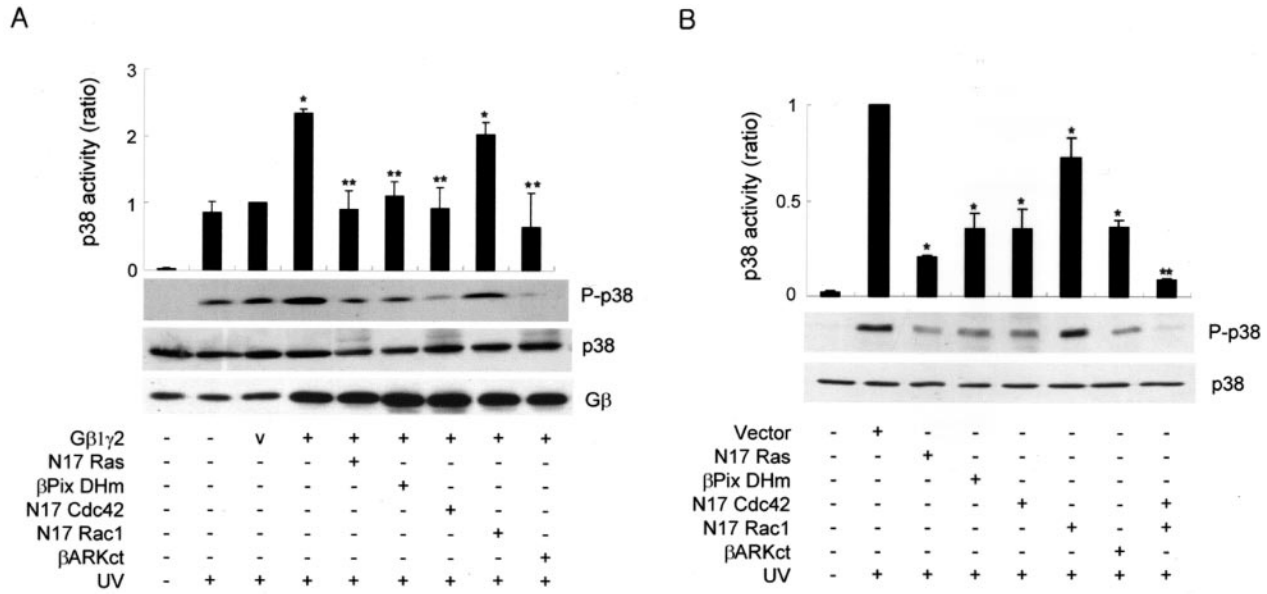

FIG. 7. **Mediation of UV-induced activation of p38 by Gβγ in HaCaT human keratinocytes.** Inhibition of UV-induced p38 activation by dominant negative Ras, βPix, Cdc42, and Rac1 in the presence (*A*) and absence (*B*) of Gβγ overexpression in HaCaT cells. HaCaT human keratinocytes were transfected with N17 Ras, βPix DHm, N17 Cdc42, N17 Rac1, or βARKct by electroporation. After 48 h, the cells were exposed to UV irradiation (100 J/m^2), and the activity of p38 was determined by Western blot using an antibody specific to phosphorylated p38. The blots shown are representative of at least 3–5 independent experiments, and the histograms represent the average and standard deviations. The asterisk (*) represents a statistically significant difference *versus* the vector-transfected control (*V*), and ** represents a statistically significant difference from Gβγ-transfected cells (*A*) or from N17 Cdc42- or Rac1-transfected cells (*B*) ($p < 0.05$, Mann-Whitney *U* test).

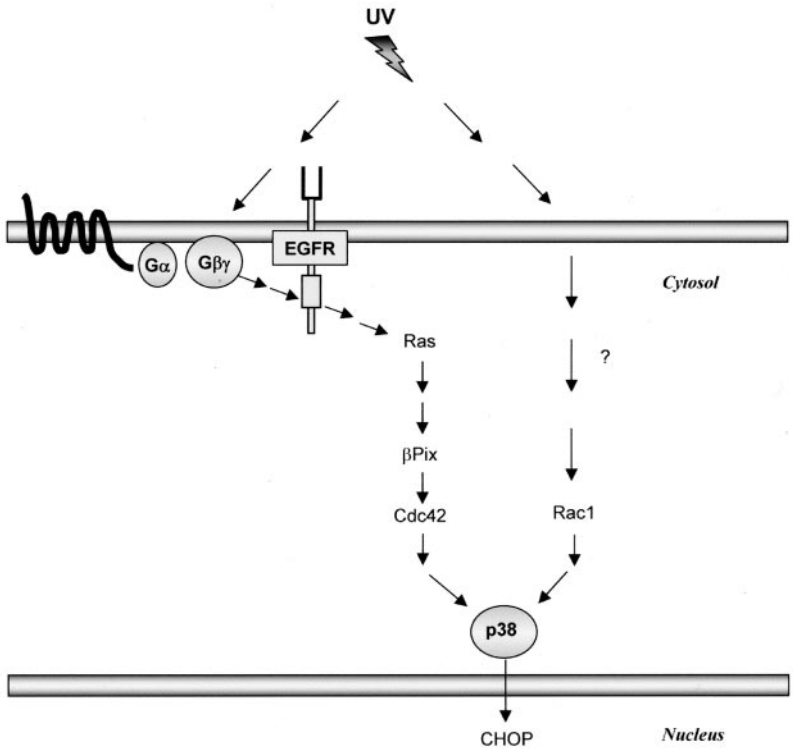

FIG. 8. **A suggested model for the UV-induced activation of p38 via the Gβγ-dependent and the Gβγ-independent pathways.**

the effect of Ras overexpression on the UV-induced activation of p38 were analyzed. Ras activity started to increase at 5 min and peaked at 10 min. This level was maintained until 40 min after UV irradiation, when Ras activity was assessed by analyzing its binding to the Ras binding domain of Raf-1 (Fig. 3*A*). $G\beta_1\gamma_2$ overexpression resulted in a 2-fold increase in UV-induced Ras activity compared with the vector-transfected COS-1 cells (Fig. 5*A*). Overexpression of the constitutively active mutant V12 Ras increased UV-induced p38 activation to 1.8 times that of the vector control, and the co-expression of V12 Ras and Gβγ did not further augment UV-induced p38 activation from that induced by Gβγ expression (Fig. 5*B*). Overexpression of V12 Ras alone without UV irradiation increased the p38 activity to 35% of that of the UV-irradiated cells (Fig. 5*C*). In contrast, the overexpression of dominant negative N17 Ras blocked most of the increase in UV-induced p38 activity that resulted from the overexpression of $G\beta_1\gamma_2$ in COS-1 cells (Fig. 5*B*), and the overexpression of N17 Ras resulted in an 80% inhibition of UV-induced p38 activity when the phosphorylation of transfected FLAG-tagged p38 was analyzed (Fig. 5*D*). In

addition, pretreatment with a Ras farnesyltransferase inhibitor, manumycin A, also blocked UV-induced p38 activity completely in the absence or presence of Gβγ overexpression (Fig. 5*E*). This result showed that Ras is involved in the UV-induced activation of p38 mediated by endogenous Gβγ as well as by exogenous Gβγ. It confirms that Ras mediates the UV-induced activation of p38, which was expected from the fact that UV irradiation activates Ras (33) and p38 (35) and that Ras is involved in the activation of Cdc42 and p38. In addition, Ras has been reported to activate p38 when cells are stimulated by growth factors (36), cytokines, and mechanical stress (37). This suggests that Ras might elicit cellular responses to various stimuli by regulating both p38 activity and ERK activity. Additionally, it is speculated that Ras might be involved not only in the UV-induced p38 activation mediated by Gβγ but also in the GPCR agonist-induced p38 activation mediated by Gβγ.

βPix binds and regulates PAK activity (38). Ras also directly activates PAK and sustains cell transformation in many cells (39). However, it is not clear whether Ras acts upstream of βPix in the UV-induced activation of p38. We investigated this issue by analyzing UV-induced p38 activation after co-transfecting constitutively active V12 Ras with dominant negative βPix in COS-1 cells. A dominant negative mutant of βPix, βPix DHm, blocked the UV-induced p38 activation increased by the overexpression of constitutively active V12 Ras (Fig. 5*F*). This result indicates that Ras might mediate UV-induced p38 activation in a βPix-dependent manner and that Ras might act upstream of βPix in the UV-induced p38 activation mediated by Gβγ in COS-1 cells. This finding is supported indirectly by a report that showed that the βPix-PAK interaction is essential for a v-Ha-Ras induced malignant transformation (40). Therefore, Ras might regulate PAK activation via βPix to induce cellular responses to a variety of signals.

The Phosphorylation of EGFR Is Involved in the UV-induced Activation of p38 Mediated by Gβγ—Because exposure to UV light induces the phosphorylation of EGFR and the activation of Ras (11, 41), we examined whether EGFR phosphorylation is involved in the UV-induced activation of p38 mediated by Gβγ. The phosphorylation of EGFR began to increase sharply at 5 min and then continued to increase until 40 min after UV irradiation (Fig. 3*A*). This time course of EGFR phosphorylation was similar to the time courses of Ras, Cdc42, and p38 activation, but differed from that of Rac1 activation. The overexpression of Gβγ proteins increased the UV-induced phosphorylation of EGFR, and treatment with AG1478, a specific inhibitor of EGFR kinase, decreased the UV-induced phosphorylation of EGFR and the activation of p38 in the presence or absence of Gβγ overexpression (Fig. 6). This result indicates that the phosphorylation of EGFR is involved in the UV-induced activation of p38 mediated by Gβγ and that Gβγ mediates UV-induced p38 activation through the activation of EGFR.

EGFR transactivation by G proteins has been identified as a critical element in GPCR-induced mitogenic signaling (42). Much evidence supports the existence of substantial integration and cooperation between GPCR and the receptor tyrosine kinase (RTK) signaling pathways, and GPCRs appear to use RTKs as signaling intermediates to mediate cell growth. The cross-talk between G protein and the receptor tyrosine kinase signaling pathways allows cells to integrate information from many different sources, thus allowing them to facilitate delicate control over cellular regulatory systems (43). The Gβγ-dependent EGFR phosphorylation identified in this study might be another example of such a transactivation of an RTK by GPCR, and it may enable GPCR to modify cellular stress responses via RTK signaling pathways.

Gβγ Also Mediates UV-induced p38 Activation in HaCaT Human Keratinocytes—To verify the physiological significance of the Cdc42-dependent mediation of UV-induced p38 activation by the Gβγ observed in COS-1 cells, we analyzed the role of the signaling molecules in UV-induced p38 activation in the human HaCaT keratinocyte cell line (44). The keratinocyte is a major component of skin, and it responds to UV irradiation in various ways by inducing inflammation, photoaging, and carcinogenesis. Overexpression of Gβγ increased UV-induced activation of p38 by ~2.3-fold, and the overexpression of βARKct that sequesters the free Gβγ complex decreased the p38 activity by ~65–70% in the presence or absence of Gβγ overexpression. In addition, overexpression of N17 Ras, βPix DHm, N17 Cdc42, and of N17 Rac1 all obviously reduced the UV-induced p38 activation in the absence and presence of Gβγ overexpression (Fig. 7, *A* and *B*). This result shows that UV-induced p38 activation mediated by Gβγ involves Ras, βPix, and Cdc42 in sequence in the human HaCaT keratinocyte cell line in the same ways observed in COS-1 cells. This implies that the mediation by Gβγ of UV-induced p38 activation via EGFR, Ras, βPix, and Cdc42 might have some physiological roles in UV-induced cellular responses. It is supported by a recent report that the UV-induced activation of p38 enhanced the resistance of normal human keratinocytes to apoptosis by stabilizing cytoplasmic p53, suggesting that p38/p53 pathway plays a key role in the adaptive response of normal keratinocytes against UV (23).

The G proteins are involved in regulating various biological functions such as metabolism, neurotransmission, secretion, and gene expression. The G proteins can also regulate a cell's fate by participating in proliferation, differentiation, or apoptosis via the activation of various MAPKs like ERK, JNK, and p38. Depending on the cell type and the GPCR stimulated, p38 activation can be mediated by Gα subunits, Gβγ subunits, or a combination of the two (5). Although, to date, there is no GPCR known to be activated by stress, our previous study showed that Gβγ mediates the p38 activation induced by UV irradiation, suggesting that the G protein might be involved in the regulation of cellular responses elicited by non-GPCR agonists (22).

In this study, we investigated the signaling pathway that connects Gβγ to the UV-induced activation of p38 in COS-1 and HaCaT cells. From this study, we conclude that signaling molecules such as Cdc42, βPix, Ras, and EGFR, are involved in the UV-induced activation of p38 mediated by Gβγ, which indicates that Gβγ can mediate UV-induced p38 activation by the sequential activation of EGFR, Ras, βPix, Cdc42, and MKK3/6. We also suggest that UV activates p38 via at least two separate pathways in cells, *i.e.* one for the immediate activation of p38, which is Rac-dependent but Gβγ-independent, and the other for the delayed and sustained activation of p38, which is Gβγ-Ras-βPix-Cdc42-dependent (Fig. 8).

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