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Acidic Extracellular pH Induces Matrix Metalloproteinase-9 Expression in Mouse Metastatic Melanoma Cells through the Phospholipase D-Mitogen-activated Protein Kinase Signaling

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Abstract: The extracellular pH (pH*e*) of tumor tissues is often acidic, which can induce the expression of several proteins. We previously showed that production of matrix metalloproteinase-9 (MMP-9) was induced by culturing cells at acidic pH*e* (5.4-6.5). Here we have investigated the signal transduction pathway by which acidic pH*e* induces MMP-9 expression. We found that acidic pH*e* (5.9) activated phospholipase D (PLD), and inhibition of PLD activity by 1-butanol and Myr-ARF6 suppressed the acidic pH*e*-induced MMP-9 expression. Exogenous PLD, but not phosphatidylinositol-specific PLC or PLA_2, mimicked MMP-9 induction by acidic pH*e*. Western blot analysis revealed that acidic pH*e* increased the steady-state levels of phosphorylated extracellular signal-regulated kinases 1/2 and p38 and that the PLD inhibitors suppressed these increases. Using 5'-deletion mutant constructs of the MMP-9 promoter, we found that the acidic pH*e*-responsive region was located at nucleotide -670 to -531, a region containing the NFκB binding site. A mutation into the NFκB binding site reduced, but not completely, the acidic pH*e*-induced MMP-9 promoter activity, and NFκB activity was induced by acidic pH*e*. Pharmacological inhibitors specific for mitogen-acti-vated protein kinase kinase 1/2 (PD098059) and p38 (SB203580) attenuated the acidic pH*e*-induced NFκB activity and MMP-9 expression. These data suggest that PLD, mitogen-activated protein kinases (extracellular signal-regulated kinases 1/2 and p38), and NFκB mediate the acidic pH*e* signaling to induce MMP-9 expression. A transcription factor(s) other than NFκB may also be involved in the MMP-9 expression.

The abbreviations used are: MMP, matrix metalloproteinase; NFκB, nuclear factor-κB; AP-1, activator protein-1; IL, interleukin; PLD, phospholipase D; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MEK, MAPK kinase; pH*e*, extracellular pH; VEGF, vascular endothelial cell growth factor; PtBut, phosphatidylbutanol; PI-PLC, phosphatidylinositol-specific phospholipase C; PC-PLC, phosphatidylcholine-specific phospholipase C; PLA_2, phospholipase A_2; SLO, streptolysin O; CM, conditioned medium; RT, reverse transcription; PBS, phosphate-buffered saline; nt, nucleotide(s).

INTRODUCTION

Proteolytic degradation of extracellular matrix is important for tumor metastasis and angiogenesis. The matrix metalloproteinases (MMPs) constitute a family of extracellular matrix-degrading enzymes. Among these enzymes, MMP-9/gelatinase B plays an important role in tumor invasion and metastasis because of its specificity for type IV collagen. Because the promoter region of the MMP-9 gene contains a TATA box and nuclear factor-κB (NFκB) and activator protein-1 (AP-1) binding sites, expression of MMP-9 mRNA can be up-regulated by stimuli such as interleukin (IL)-l and tumor necrosis factor-α (1). In addition, MMP-9 expression can be up-regulated by phorbol 12-O-tetradecanoate 13-acetate through phospholipase D (PLD) (2, 3).

Mitogen-activated protein kinases (MAPKs) are crucial enzymes in the receptor-mediated signaling cascade. There are three major groups of MAPKs, extracellular signal-regulated kinase (ERK) 1/2, p38, and c-Jun N-terminal kinase (JNK) 1/2. PD98059 and SB203580, which are specific inhibitors of MAPK kinase (MEK) 1/2 and p38, respectively, repress MMP-9 expression and *in vitro* tumor cell invasion (4, 5). Moreover, the JNK inhibitor SP600125 has been shown to attenuate phorbol 12-*O*-tetradecanoate 13-acetate-induced MMP-9 expression (6).

The extracellular pH (pH*e*) of solid tumors is acidic due to the presence of anaerobic glucose metabolites such as lactate. For example, the basal pH*e* of xenografted human tumors was shown to be acidic, and this acidity was increased after intravenous injection of D-glucose (7). Using glycolysis-impaired (phosphoglucose isomerase-deficient) cells, it was recently shown that, in addition to lactate, CO_2 was a significant source of acidity in tumors through the pentose phosphate pathway (8). These observations suggest that acidity in tumors can be caused by hypoxia-dependent and/or-independent pathways.

Clinically, the acidic pH*e* of solid tumors has been found to modulate their sensitivity to radiation and chemotherapeutic agents (9-13). We have shown that in mouse metastatic B16 melanoma cells, expression of MMP-9 and *in vitro* invasiveness are induced by acidic pH*e* (pH*e* 5.4-6.5) (14). In addition, acidic pH*e* increases the expression of platelet-derived endothelial cell growth factor/thymidine phosphorylase in human breast tumor cells (15), of the inducible isoform of nitric oxide synthase (iNOS) in macrophages (16), of vascular endothelial cell growth factor (VEGF) in glioma (17) and glioblastoma (18) cells, and of IL-8 expression in human pancreatic adenocarcinoma (19-21) and ovarian carcinoma (22) cells through activation of the transcription factors NF-κB and/or AP-1. These facts suggest that microenvironmental acidic pH*e* stimulates tumor growth and angiogenesis and favors metastasis. Although acidic pH*e* has been shown to activate Ras and the ERK1/2 pathway to enhance VEGF transcription via AP-1 (18), the mechanism by which acidic pH*e* regulates gene expression, including MMP-9, has poorly been understood. We, therefore, further investigated acidic pH*e* signaling in mouse B16 melanoma cells. Because the optimal MMP-9 induction was observed at pH*e* 5.4-6.5, whereas the optimum pH*e* for cell growth rate was around 6.8 (14), we fixed pH*e* at 5.9 to investigate the intracellular signaling pathways for MMP-9 expression.

MATERIALS AND METHODS

Reagents

Myristoylated N-terminal domain peptide (amino acid residues 2-13) of ADP-ribosylation factor 6 (Myr-ARF6) (a PLD inhibitor), SB203580 and SB202190 (p38 inhibitors), and PD98059 (a MEK1/2 inhibitor) were purchased from Calbiochem. Dulbecco's modified Eagle's medium/F-12 (1:1 mixture) and Lipofectamine™ 2000 were purchased from Invitrogen, dual luciferase reporter assay kits were from Toyo Ink (Tokyo, Japan), and cabbage PLD, *Bacillus cereus* phos-phatidylinositol-specific phospholipase C (PI-PLC) and phosphatidyl-choline-specific phospholipase C (PC-PLC), *Apis mellifera* phospholipase A_2 (PLA_2), and streptolysin O (SLO) were from Sigma. Fetal bovine serum was purchased from Cell Culture Technologies GmbH (Zurich, Switzerland); Immobilon-P membrane was from Millipore (Bedford, MA); nuclear extract kits were from Active Motif (Carlsbad, CA); Instra-Pure RNA purification kits were from Eurogentic (Liege, Belgium); Immunostar™ Western blotting detection kits, which included a chemiluminescent agent and peroxidase-conjugated swine anti-rabbit IgG or peroxidase-conjugated goat anti-mouse IgG, were from Wako (Tokyo, Japan). The blocking reagent N102 was obtained from NOF Corp. (Tokyo, Japan); antibodies directed against total or phos-phorylated MAPKs were from Santa Cruz (Santa Cruz, CA); [9,10-^{3}H]palmitic acid (50.0 Ci/mmol) was from Moravec Biochemicals (Brea, CA); phosphatidylbutanol (l-*O*-palmitoyl-2- *O* -oleoyl-sra-3-phosphobu-tanol; PtBut) was from Biomol Research Laboratories (Plymouth Meeting, PA); [*methyl*-^{3}H]choline chloride (83.0 Ci/mmol) was from Amersham Biosciences (Piscataway, NJ); phosphorylcholine was from Tokyo Kasei Kogyo (Tokyo, Japan); GeneAmp Thermostable rTth transcriptase RNA PCR kits were from PerkinElmer Life Sciences (Boston, MA); GelStar was from FMC BioProducts (Rockland, ME).

Vectors

The PathoDetect® NFκB and AP-1 cis-reporting system (pNFκB-Luc, pAP1-Luc) was purchased from Stratagene (La Jolla, CA). The luciferase reporter gene constructs driven by the 5 '-flanking region of human MMP-9 (-670, -530, -73) were the kind gifts of Dr. Douglas D. Boyd (MD Anderson Cancer Center, University of Texas, TX) with the permission of Dr. Motoharu Seiki (Institute of Medical Science, University of Tokyo, Tokyo, Japan) who originally constructed it as a chloramphenicol acetyltransferase reporter vector (6, 23, 24). The luciferase reporter gene constructs driven by the 5'-flanking region (-681 to +63) of mouse MMP-9 (pGL3MMP-9) and by the mutant MMP-9 promoter containing a double point mutation (C → G) in the NFκB binding site (pGL3MMP-9ΔNFκB) were the kind gifts of Dr. Yves St-Pierre (INRS-Institut Armand-Frappier, Université du Québec, Québec, Canada) (25). The cytomegalovirus-driven *Renilla* luciferase reporter vector (pRL-CMV, Promega, Madison, WI) was used to monitor transfection efficiency.

Cells and Cell Culture

B16-BL6 cells were cultured in Dulbecco's modified Eagle's medium containing 10mM HEPES (pH 7.3) supplemented with 10% fetal bovine serum. As reported previously (14), maximum induction of MMP-9 expression was in the pH*e* range of 5.4-6.5. We, therefore, used serum-free medium at pH*e* 5.9 as an experimental model of extracellular acidification. To prepare serum-free assay media, Dulbecco's modified Eagle's medium/F-12 was supplemented with 15 mM HEPES and 4 mM phosphoric acid and adjusted pH to 5.9 with HC1 (acidic pH*e*) or to 7.3 with NaOH (neutral pH*e*) (14, 26).

Permeabilization with SLO and Inhibitor Treatment

Permeabilization for Myr-ARF6 was performed with SLO as described (27, 28), with some modifications. Briefly, cells were washed twice with PBS, incubated for 5 min with 15 units/ml SLO dissolved in PBS, and cultured with Myr-ARF6. In some experiments, Myr-ARF6 treatment was performed in the absence of cell permeabilization with SLO, since the latter did not significantly affect Myr-ARF6 inhibition of PLD and acidic pH*e*-induced MMP-9 expression. The MAPK inhibitors, SB203580, SB202190, and PD98059 and Myr-ARF6 were dissolved in dimethyl sulfoxide (Me_2SO), and these stock solutions were stored at -30 °C and diluted with assay medium just before the experiment. The final concentration of Me_2SO was less than 0.2%. As a control, cells were treated with vehicle alone.

Preparation of Conditioned Medium (CM) for Zymography

Proteins in CM were precipitated with 3 volumes of ethanol for at least 3 h at -80 °C and collected by centrifugation at 15,000 x *g* for 30 min at 4 °C (29). After drying at room temperature, the precipitates were dissolved in PBS or mixed directly with SDS sample buffer (0.5 mM Tris-HCl (pH 6.8), 1% SDS, 0.2% glycerol). The quantity of samples was normalized for zymography based on the DNA contents of the cultures (1.5 μg of DNA/lane; ≈ 10 μg of protein/lane), as measured using bisbenzimide (30). Protein concentration was determined by the Bradford method (Bio-Rad protein assay kit, Hercules, CA) using bovine serum albumin as the standard.

Gelatin Zymography

Gelatinolytic activities were analyzed by gelatin zymography as described (14, 31, 32). Briefly, ethanol-precipitated proteins were subjected to SDS-PAGE in 7.5% polyacrylamide gels containing 0.1% gelatin. The gels were washed with 2.5% Triton X-100 at room temperature with gentle shaking for 1 h and incubated for 20 h in reaction buffer (50 mM Tris-HCl (pH 7.5), l00mM NaCl, 10mM$CaCl_2$, 0.002% NaN_3) at 37 °C. Gelatinolytic activity was visualized by Coo-massie Brilliant Blue R250 staining.

Western Blot Analysis

The active forms of MAPKs were visualized and quantified by Western blotting. Cells were lysed with the nuclear extract Kit according to the manufacturer's protocol. Proteins in the cell lysate (20 μg) were separated by 10% SDS-PAGE and transferred to Immobilon-P membranes using the Bio-Rad Western blot apparatus. After blocking with 5-times diluted blocking reagent N102 in Tris-buffered saline solution (20 mM Tris-HCl (pH 7.6), 137 mM NaCl) containing 0.05% Tween 20, the membrane was incubated with primary antibody in Tris-buffered saline solution containing 0.05% Tween 20 and 10% blocking reagent N102. After incubation with an appropriate secondary, horseradish peroxidase-conjugated antibody, an Immunostar™ detection kit with a chemiluminescent substrate followed by exposure to x-ray film was used to detect binding.

Determination of PLD and PC-PLC Activities

Cells washed twice with ice-cold PBS were lysed with 0.2% Triton X-100, and their protein concentrations were determined. PLD and PC-PLC activities were detected using the Amplex™ Red assay kit (Molecular Probes, Eugene, OR) (33). For the PLD assay, the lysates were incubated with 250 μM PC, 100 milliunits/ml choline oxidase derived from *Alcaligenes* sp., 1 unit/ml horseradish peroxidase, and 50 μM 10-acetyl-3,7-dihydrophenoxazine (Amplex™ Red reagent) in a reaction buffer consisting of 50 mM Tris-HCl (pH 8.0), 5 mM $CaCl_2$, and 0.2% Triton X-100. PLD activity was measured with a fluorescence microplate reader using an excitation wavelength of 535 nm and a detection wavelength of 590 nm. In some experiments cell lysates were incubated with the same mixture but excluding PC to detect endogenous choline.

For the PC-PLC assay, phosphorylcholine was treated with alkaline phosphatase to produce choline before the oxidase reaction step. That is, the cell lysates were incubated with 500 μM PC, 4 units/ml alkaline phosphatase, 100 mM choline oxidase, 1 units/ml horseradish peroxidase, and 200 μM Amplex™ Red reagent in a reaction buffer consisting of 50 mM Tris-HCl (pH 7.4), 140 mM NaCl, 10 mM dimethylglutarate, 2 mM $CaCl_2$, and 0.2% Triton X-100. PC-PLC activity was measured with a fluorescence microplate reader as above. In some experiments production of radiolabeled phosphorylcholine was determined as the *in vivo* PC-PLC activity according to Zamorano *et al.* (34) with some modifications. Briefly, cells in 6-well plates were labeled with 1 μCi/ml of [*methyl*-^{3}H] choline chloride in serum-free neutral pH medium for 24 h. They were washed twice with warmed PBS and incubated with neutral or acidic assay medium for the indicated time. The reaction was stopped by adding methanol/H_2O (1:1, v/v). The cell suspension in methanol/H_2O was mixed with equal volume of chloroform. The upper (water) phase was then transferred into a new glass tube, dried, and separated by thin-layer chromatography using a silica gel 60 plate (Merck) and methanoyH_2O/NH_4OH (100:100:2, v/v/v/v) as a solvent. [^{3}H] Phosphorylcholine formed was identified by co-migration of authentic phosphorylcholine. The spots corresponding to phosphorylcholine, which were revealed by iodine vapors, were scraped off and put into scintillation vials. The radioactivity was counted by liquid scintillation counting (35).

In vivo transphosphatidylation activity of PLD was determined according to Banno *et al.* (36) with some modifications. Cells in 6-well plates were labeled with 1 μCi/ml [9,10-^{3}H]palmitic acid in serum-free neutral pH medium for 18 h. They were washed twice with warmed PBS and incubated with neutral or acidic assay medium for 24 h in the presence of 0.3% 1-butanol. Lipids were extracted from cells with chlo-roform/methanol (2:1, v/v), collected from lower (chloroform) phase, and separated by thin-layer chromatography using a silica gel 60 plate and an upper phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/H_2O (13:2:3:10, v/v/v/v) as a solvent. [^{3}H]PtBut formed was identified by co-migration of authentic PtBut (l-*O*-palmitoyl-2- *O* -oleoyl-sn-3-phos-phobutanol). The spots corresponding to PtBut, which were revealed by iodine vapors, were scraped off and put into scintillation vials. The radioactivity was counted by liquid scintillation counting as described above.

Quantitative Reverse Transcription (RT)-PCR

MMP-9 mRNA expression was measured by quantitative RT-PCR using a synthetic RNA as the internal standard (37). Total RNA (10 ng) was extracted from cells using an Instra-Pure RNA purification kit, and the RNA was reverse-transcribed and amplified by a GeneAmp Thermostable rTth transcriptase RNA PCR kit. The amplification primers for MMP-9 were 5'-GTTTTTGATGCTATTGCTGAGATCCA-3' (forward) and 5'-CCCA-CATTTGACGTCCAGAGAAGAA-3' (reverse), which yielded a 208-bp product for endogenous mRNA and a 271-bp product for multistandard synthetic RNA. As a control, we amplified a 28 S rRNA sequence using the primers, 5'-GTTCACCCACTAATAGGGAACGTGA-3' (forward) and 5'-GGATTCTGACTTAGAGGCGTTCAGT-3' (reverse), which yielded a 212-bp product for endogenous mRNA and a 269-bp product for multistandard synthetic RNA. The amplification protocol consisted of 35 cycles (for MMP-9) or 19 cycles (for 28 S rRNA) of denaturation at 94 °C for 15 s, annealing at 66 °C for 20 s, and extension at 72 °C for 10 s. The PCR products were separated by 10% polyacrylamide gels, stained with GelStar, and quantified using a Fluor-S-Multilmager (Bio-Rad).

Luciferase Reporter Assay

To detect NFκB, AP-1, and MMP-9 promoter activities, we performed luciferase reporter gene assays with pNFκB-Luc, pAP-1-Luc, and MMP-9 promoter constructs (the 5'-deletion mutant and the NFκB-site mutant), respectively. These reporter vectors (1 μg) were transfected into B16-BL6 cells with Lipofectamine™ 2000 in 6-well culture plates according to the manufacturer's protocol. Transfection efficiency was monitored by co-transfection of *Renilla* luciferase reporter vector (pRL-CMV). The reporter activity was measured using dual luciferase reporter assay kits.

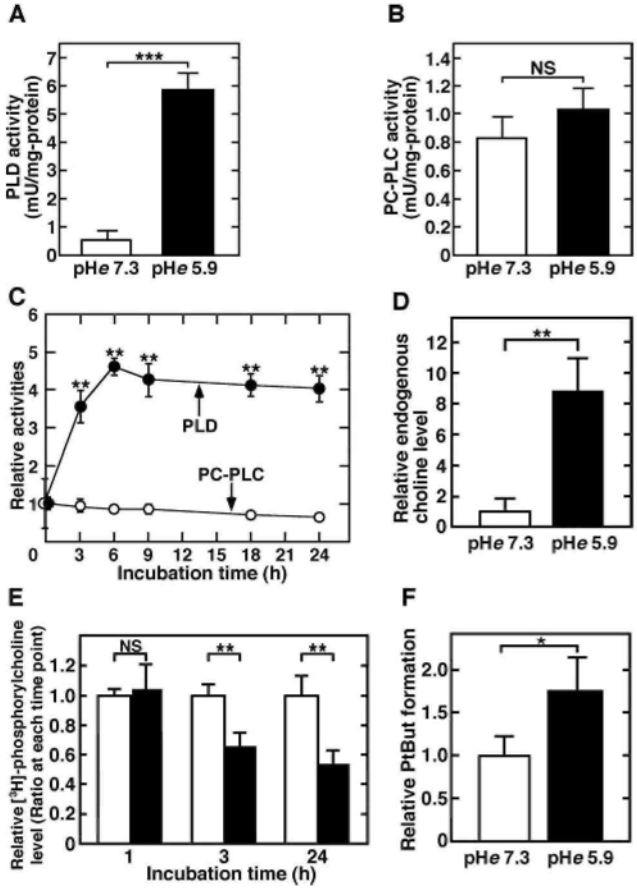
RESULTS

PLD Mediates MMP-9 Expression by Acidic pH*e*

Because activation of PLD has been shown to be essential for induction of MMP-9 expression by several stimuli (2, 3), we first assayed PLD activity after shifting pH*e* to an acidic pH. As expected, when cells were incubated at pH*e* 5.9, the PLD activity was significantly higher than that of the cells incubated at pH*e* 7.3 (Fig. LA). Because PC metabolism might be involved in acidic pH*e* signaling, we assayed PC-PLC activity, but it was not significantly affected by acidic pH*e* (Fig. 1B). When the time course of PLD activation after shifting pH*e* to acidic pH was examined, this activity increased to 80% of the maximum level at 3 h and reached a plateau at 6 h

that lasted up to 24 h ($p < 0.01$) (Fig. 1C). The level of endogenous choline, the metabolite of PLD, was also higher in cells cultured at acidic pH*e* than those at neutral pH*e* (Fig. 1D). Whereas the phosphorylcholine level was not affected by acidic pH*e* during a 1-h incubation (Fig. 1*E*), it rather decreased by further incubation. We also determined *in vivo* transphosphatidylation activity of PLD. The PtBut level was significantly higher in the acidic pH*e*-treated cells (Fig. 1*F*). Taken together, these results show that PLD, but not PC-PLC, was activated by acidic pH*e*.

FIG. 1: *PLD, but not PC-PLC, activity increases in cells cultured at acidic pHe.*
Nearly confluent cultures in a 60-mm diameter dish were treated for 24 h (A and B) or the indicated time (C) with serum-free media at pH 5.9. The cells were lysed with 0.2% Triton X-100, and PLD (A and C) and PC-PLC (B and C) activities were measured by the Amplex™ Red assay kit. mU, milliunits. NS, not significant. C, the time course of PLD and PLC activity after the shift to acidic pH*e*. D, endogenous choline level after the shift to acidic pH*e*. Four hours after the shift to acidic pH*e*, the cells were lysed with 0.2% Triton X-100 and incubated with the Amplex™ Red PLD assay reaction mixture excluding PC. E, phosphorylcholine formation in neutral (*open column*) and acidic pH*e*. [methyl-^{3}H]Choline-labeled cells were incubated in neutral or acidic pH*e* (*closed column*). After the indicated time, radioactivities of [^{3}H] phosphorylcholine formed were counted by liquid scintillation counter. F, PtBut formation in neutral and acidic pH*e*. [9,10-^{3}H]Palmitic acid-labeled cells were cultured with neutral or acidic pH medium in the presence of 0.3% 1-butanol for 24 h. Radioactivities of [^{3}H] PtBut formed were counted by liquid scintillation counter. NS, not significant. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.



To establishment the involvement of PLD in the signaling pathway, B16-BL6 cells cultured in serum-free medium at pH 5.9 were treated with 1-butanol, an inhibitor of PLD. This PLD inhibitor dose-dependently suppressed MMP-9 expression induced by acidic pH*e*, with an IC_{50} of between 0.06 and 0.13% (Fig. 2A), in agreement with previous findings (38). We also tested the effect of 2-butanol as a control, which is not a substrate of PLD. However, this reagent was too toxic to the cells for comparison; a significant cytotoxic dose was 1% for 1-butanol and 0.25% for 2-butanol. To establish the contribution of PLD to acidic pH*e* signaling, we used a synthetic myristoylated peptide, which corresponded to the N-terminal domain (amino acid residues 2-13) of ARF6 (Myr-ARF6). This reagent also suppressed MMP-9 induction (Fig. *2B),* with an IC_{50} of about 12.5 μM, in agreement with previous results (28). Because Myr-ARF6 has been generally used with SLO-treated cells (27, 28), we checked whether Myr-ARF6 could inhibit PLD activity in the absence of SLO treatment. We found that Myr-ARF6 attenuated acidic pH*e*-induced PLD activity to 39.8 ± 4.15% in the presence of SLO treatment before shifting pH*e* (Fig. 2C). When cells were treated with Myr-ARF6 in the absence of SLO treatment, PLD activity decreased to 46.0 ± 1.63% (Fig. 2C).

Thus, SLO treatment did not significantly affect Myr-ARF6 inhibition of acidic pH*e*-induced PLD activity in these cells. Zymographic analysis also showed no significant difference between the presence and absence of SLO treatment on Myr-ARF6 inhibition of acidic pH*e*-induced MMP-9 production (Fig. *2D*). We also found that incubation with Myr-ARF6 for only 10 min before shifting pH*e* resulted in significant reduction of MMP-9 induction (Fig. 2D). In addition, when PLD was added into the culture medium at neutral pH*e*, it mimicked MMP-9 induction by acidic pH*e*, whereas PI-PLC and PLA2 did not (Fig. 3). Thus, these results support our hypothesis that PLD is an important enzyme for acidic pH*e* regulation of MMP-9 expression.

FIG. 2: *Inhibition of PLD activity suppresses acidic pHe-in-duced MMP-9 expression.*
A-B, inhibition of acidic pH*e*-induced MMP-9 expression by PLD inhibitors. Nearly confluent cultures in a 24-well culture plate were incubated for 48 h with the indicated concentrations of 1-butanol (A) or Myr-ARF6 peptide (B) at pH*e* 5.9. CM was collected, concentrated, and analyzed by zymography to detect MMP-9. Arrowhead, pro-MMP-9. C, inhibition of PLD activity by Myr-ARF6 peptide. Nearly confluent cultures in a 60-mm diameter dish were incubated for 5 min with or without 15 units/ml SLO followed by incubation for 24 h with 25 μM Myr-ARF6 peptide at pH*e* 5.9. The cells were lysed with 0.2% Triton X-100, and their PLD activity was determined by the Amplex™ Red assay kit. NS, not significant. D, inhibition of MMP-9 expression by Myr-ARF6 peptide. Nearly confluent cultures in a 24-well plate were treated for 5 min with or without 15 units/ml SLO followed by incubation for 48 h with 25 μM Myr-ARF6 peptide at pH*e* 5.9 (Syn-Myr-ARF6) or for 10 min with 25 μM Myr-ARF6 peptide at pH*e* 7.3 and then for 48 h at pH*e* 5.9 in the absence of Myr-ARF6 peptide (Pre-Myr-ARF6). CM was collected, concentrated, and analyzed by zymography to detect MMP-9. Arrowhead, pro-MMP-9.

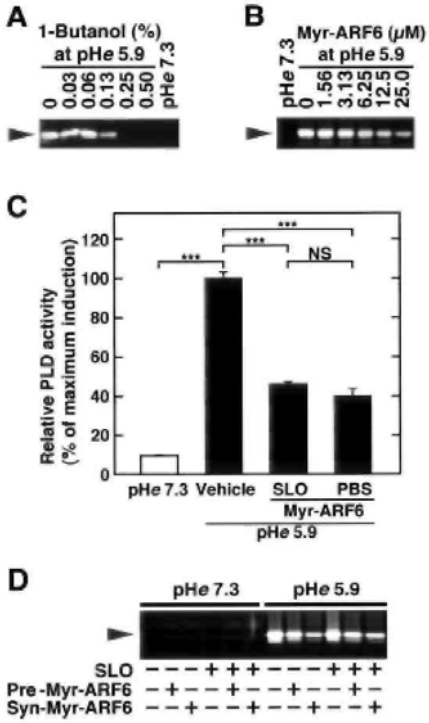
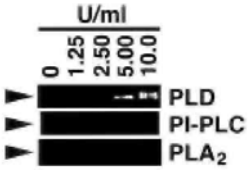


FIG. 3: *Exogenous PLD, but not PI-PLC or PLA_2, induced MMP-9 expression at neutral pHe.*
Cells were incubated for 24 h with the indicated concentrations of PLD, PI-PLC, or PLA_2 at pH*e* 7.3. CM was collected, concentrated, and analyzed by zymography to detect MMP-9. *U,* units. *Arrowheads,* pro-MMP-9.



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ERKs and p38 but Not JNKs Are Downstream Effectors of PLD in Acidic pH*e* Signaling

To assess the contribution of MAPKs as the downstream targets of PLD, we assayed the level of the phosphorylated, or active, forms of the MAPKs. Western blotting using antibodies specific for phosphorylated MAPKs showed that ERK1/2 and p38 were present in their inactive forms in cells cultured at neutral pH*e*, but the levels of phosphorylated ERK1/2 and p38 significantly increased at acidic pH*e* (Fig. 4). In contrast, the only active form of JNKs observed was JNK1, and the level of this isoform was not affected by acidic pH*e*. The PLD inhibitors 1-butanol and Myr-ARF6 attenuated the phosphorylation of both ERK1/2 and p38, but they did not affect the total amounts of ERK1/2 and p38. In contrast, these inhibitors had no effect on the level of either total or phosphorylated JNK1. Taken together, these data suggest that ERK1/2 and p38 are downstream targets of PLD.

We further tested this hypothesis by assaying the effects of the pharmacological inhibitors of p38 (SB203580) and of MEK (PD980598). Each of these specific inhibitors dose-dependently suppressed MMP-9 induction at acidic pH*e* (Fig. 5A). Similarly, a second inhibitor of p38, SB202190, also inhibited acidic pH*e*-induced MMP-9 expression (data not shown).

FIG. 4: *Western blot analysis of MAPKs.*
Cells were incubated for 24 h in acidic serum-free medium including PLD inhibitors (0.25% 1-butanol or 25 mM Myr-ARF6) without SLO treatment. The cells were lysed, and the lysates were analyzed by Western blotting using antibodies specific for phospho (*p*)-ERKl/2, p38, and JNK1/2. The membranes were subsequently stripped with mercaptoethanol and reprobed with antibodies specific for total ERK1/2, p38, and JNK1/2.

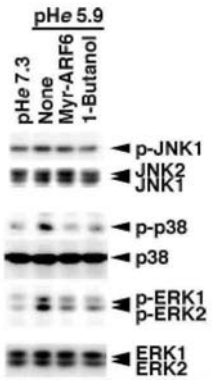
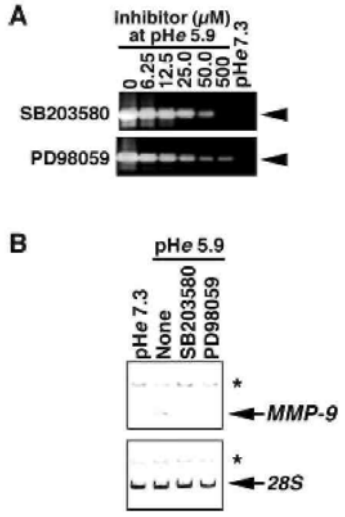


FIG. 5: *Inhibition of acidic pHe-induced MMP-9 expression by MAPK inhibitors.*
A, zymography. Cells were incubated with the indicated concentrations of MAPK inhibitors (SB203580 or PD980598) at pH*e* 5.9, and the concentrated CM was analyzed by zymography to detect MMP-9. *Arrowheads,* pro-MMP-9. *B,* quantitative RT-PCR. Cells were cultured with SB203580 (25 μM) or PD980598 (50 μM) at pH*e* 5.9 or pH*e* 7.3 (control). Total RNA was extracted and reverse-transcribed with synthetic RNA as the internal control and amplified by PCR. PCR products were electrophoresed on 10% polyacrylamide gels, which were stained with GelStar and analyzed using Fluor-S-Multilmager. *Arrows, MMP-9* and *28 S* rRNA; *, multi-standard synthetic RNA.



To determine whether the regulation of MMP-9 expression by MAPKs involves pretranslational events, we measured MMP-9 mRNA by RT-PCR after incubation of cells at neutral or acidic pH*e* in the presence or absence of SB203580 or PD980598. The efficiency of the reaction was monitored by adding identical amounts of a standard synthetic RNA to the samples. Each standard RNA was designed to be amplified by the same primers as the gene of interest but to yield reaction products of different sizes, enabling their discrimination by gel electrophoresis. Measurement of 28 S rRNA was used to standardize the actual amount of total RNA in the samples. We found that MMP-9 mRNA was barely detectable in cells at neutral pH*e* but expressed in cells at acidic pH*e*, as previously observed (26) (Fig. *5B*). Incubation of cells with SB203580 or PD980598 at acidic pH*e* reduced the level of MMP-9 mRNA almost to that observed at neutral pH*e*. Because SB203580 and PD980598 have been reported to inhibit the activity of cyclooxygenase-2, an enzyme that converts arachidonic acid to pro-inflammatory eicosanoids (39), and since cyclooxygenase-2 contributes to MMP-9 expression (40), we tested the effect of NS-398, an inhibitor specific for cyclooxygenase-2, on the acidic pH*e*-induced MMP-9 expression. NS-398 had no effect on MMP-9 expression at acidic pH*e* (data not shown). This suggested that cyclooxygenase-2 is not involved the induction of MMP-9 by acidic pH*e*.

Acidic pHe Increases NFκB Activity through ERK1/2 and p38

To determine the transcription factor involved in the acidic pH*e* signaling, we measured MMP-9 promoter activity using 5'-deletion mutants of human MMP-9 promoter-luciferase reporter constructs. A diagrammatic representation of the transcription factor binding sites is shown in Fig. 6A. When cells were transfected with a construct containing the nt -670 promoter fragment, which included both the NFκB and AP-1 sites, acidic pH*e* significantly increased promoter activity (Fig. 6*B*). Transfection of a construct containing the nt -531 promoter fragment resulted in a 33% reduction in the acidic pH*e*-induced promoter activity, to a level equal to that observed when the nt -670 construct was assayed at neutral pH*e*. Promoter activity of the nt -73 fragment, containing only the GC and TATA boxes, was negligible. These results suggested that the promoter region between nt -670 and nt -531 is essential for the acidic pH*e* induction of MMP-9 expression. Because two transcription factors, NFκB and AP-1, have been shown to be essential for cytokine induction of MMP-9 expression (23) and because the promoter region between nt -670 and -531 contains an NFκB binding site, we determined the effect of a double mutation (C → G) in NFκB binding site on the MMP-9 promoter activity using the luciferase reporter constructs of mouse MMP-9 promoter (-681 to +63). Mouse MMP-9 promoter activity showed 4.6-fold induction by acidic pH*e*, and this induction was effectively suppressed by the double mutation C → G into the NFκB binding site of MMP-9 promoter (51.8% reduction) (Fig. 6C). The induction rate of the mutant promoter activity was 3.2, whereas that of the wild type promoter activity was 4.6. Furthermore, NFκB transcriptional activity was assayed. As expected, NFκB activity was significantly induced by the acidic pH*e* treatment but suppressed by the MEK inhibitor PD098059 and by the p38 inhibitor SB203580 (Fig. 7). In contrast, AP-1 activity was negligible under all experimental conditions tested. These data suggest that the acidic pH*e* induction of MMP-9 expression occurs via NFκB activation by ERK1/2 and p38.

DISCUSSION

The pH*e* in the microenvironment of tumors is often lower than in normal tissues. We previously found that acidic pH*e* increases MMP-9 production by various B16 melanoma clones and by the human HT1080 and A549 cell lines as well as increasing the *in vitro* invasiveness of B16 melanoma cells (14, 26). Acidic pH*e* has also been shown to induce the expression of various proteins, such as VEGF and IL-8 (15-22). Because some of these acidic pH*e*-induced proteins are associated with tumor angiogenesis (15-22), microenvironmental acidic pH*e* may not only be associated with reduced efficacy of radiation therapy and chemotherapy (9-13) but may also support tumor growth, invasion, and metastasis.

Following our initial observation that MMP-9 expression was up-regulated in B16 cell clones by acidic pH*e* (26), we hypothesized that this increased MMP expression may have been induced by cytokines released from the plasma membrane or an intracellular pool by acidic pH*e* shock. This possibility was eliminated, however, because MMP-9 expression was not induced in cells incubated in a culture medium conditioned at pH 5.9 for 24 h and then adjusted to pH 7.3 (26). This observation prompted us to investigate an alternative intracellular mechanism(s) leading to the pH*e* effect on MMP-9 expression. As a first approach, we focused on PLD activation, because the latter has been shown to play a central role in MMP-9 overexpression by various stimuli (2, 3). As expected, we found that acidic pH*e* significantly increased PLD activity. In addition, inhibition of PLD activity by 1-butanol or Myr-ARF6 suppressed the induction of MMP-9 by acidic pH*e*, and the addition of PLD to the cultures at neutral pH*e* induced MMP-9 expression. Because PC-PLC activity did not increase at acidic pH*e*, exogenous PI-PLC did not induce MMP-9 induction at neutral pH*e*, and phorbol 12-O-tetradecanoate 13-acetate and other analogues of diacylglycerol did not induce MMP-9 expression at neutral pH*e*, we believe that

diacylglycerol can be ruled out as a second messenger mediating acidic pH*e* signaling. We also found that phosphorylcholine levels were distinguishable between neutral and acidic pH*e* in 1-h incubation, but they rather decreased at acidic pH*e* after 3 h, the time before the full activation of PLD (see Fig. 1). In addition, treatment of the cells with D609, an inhibitor of PC-PLC, did not suppress the acidic pH*e*-induced MMP-9 expression (data not shown). These also suggested that phosphatidate production from diacylglycerol via diacylglycerol kinase did not contribute to the acidic pH*e* signaling.

FIG. 6: *The MMP-9 promoter region between nt -670 and -531 contains the elements essential for acidic pHe-induced activation of MMP-9 transcription.*
A, diagrammatic representation of the transcription factor binding sites in the human MMP-9 promoter (23, 24). Cells were transfected with 1 μg of luciferase reporter plasmids containing 5'-deleted human MMP-9 promoter fragments (-670, -531, -73) *(B)* or mouse MMP-9 promoter (nt -681, pGL3MMP9) and its promoter containing a mutated NFκB site (pGL3MMP9ΔNFκB) (C) using Lipofectamine™ 2000. After 18 h, the cells were washed twice, cultured for 24 h in serum-free medium at pH*e* 7.3 or 5.9, and lysed, and the lysates were subjected to a dual luciferase assay. The transfection efficiency was normalized by the co-transfection of *Renilla* luciferase reporter construct. ***, $p < 0.001$ (Student's *t* test). *GT,* GT-box (GGGGTGGGG); *NS,* not significant.

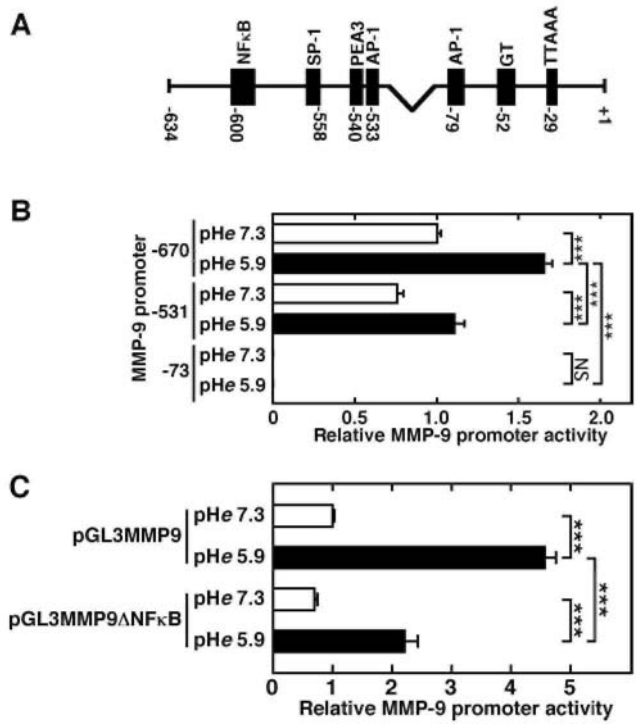
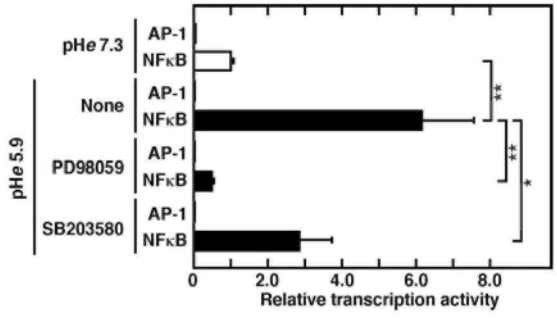


FIG. 7: *NFKB and AP-1 activities in acidic pHe-cultured cells.*
Cells were transfected with 1 μg of NFκB or AP-1 reporter constructs using Lipofectamine™ 2000. After 18 h, cells were washed twice, cultured for 48 h in serum-free media at pH 7.3 or 5.9, and lysed, and the lysates were subjected to a dual luciferase assay. The transfection efficiency was normalized by the co-transfection of *Renilla* luciferase reporter construct. *,$p < 0.05$; **,$p < 0.01$ (Student's *t* test).



Because MAPKs have been reported to be involved in regulating MMP-9 expression (5, 24, 41), we tested the possibility that MAPKs act as the downstream effectors of PLD. We found that acidic pH*e* increased the active forms of ERK1/2 and p38, but not JNK, suggesting that ERK1/2 and p38 participate in the regulation of MMP-9. This was supported by our findings with MEK1/2 and p38 inhibitors, which decreased the expression of MMP-9 induced by acidic pH*e*. Phosphorylation of p38 and ERK1/2 at acidic pH*e* was inhibited by Myr-ARF6, showing that PLD is required to elicit this effect. These data are in agreement with studies showing that PLD activates ERK1/2 in NIH 3T3 cells (42) and p38 in neutrophillike HL-60 cells (43). Although we found that Myr-ARF6 completely inhibited p38 activation, it only partially inhibited ERK1/2 activation, thus suggesting the involvement of other upstream regulators of ERK1/2 such as Ras. Indeed, activation of the Ras-ERKl/2 pathway by acidic pH*e* was shown to enhance VEGF transcription, although this signaling involved AP-1, not NF-κB, activation (18). Because JNK activation was not affected by acidic pH*e*, involvement of AP-1 in this signaling was regarded as unlikely. As expected therefore, acidic pH*e* did not increase AP-1 activity. In contrast, NFκB activity was significantly increased when the cells were cultured at acidic pH*e*, and treatment with inhibitors of MEK1/2 and p38 suppressed acidic pH*e*-induced MMP-9 expression.

Although the 5'-deletion to nt -531 from nt -670 resulted in a 33% reduction of acidic pH*e*-induced human MMP-9 promoter activity, the nt -531 promoter construct still retained the relative promoter inducibility about 1.5-fold, whereas the inducibility of nt -670 construct was 1.7-fold. When mouse MMP-9 promoter constructs were used, the induction rates of the wild-type promoter and its mutant in the NFκB-site were 4.6 and 3.2, respectively. Thus, the mutant MMP-9 promoter still retained the inducibility by acidic pH*e*. These data suggest that in addition to NFκB, another transcription factor(s) capable of binding to the promoter region between nt -670 and -531 may be involved in the acidic pH*e* signaling. Because it has been reported that NFκB and ets play pivotal roles in the signaling of IL-lβ and cell to cell contact to induce MMP-9 expression (44, 45), ets is a candidate for another transcription factor that is involved in the acidic pH*e* signaling.

Acidic pH*e* has also been shown to increase the expression of platelet-derived endothelial cell growth factor/thymidine phosphorylase (15), IL-8 (19-22), and VEGF (17, 18) in various types of cells. The IL-8 promoter contains AP1 and NFκB sites that are important for its regulation (46), and its induction is driven by p38, ERK1/2, and PLD (47, 48). VEGF expression is also induced by p38 (49), and expression of thymidine phosphorylase is regulated by p38 and ERK1/2 (50). These observations suggest that the signaling pathways described here for acidic pH*e*-induced MMP-9 expression are also relevant for these genes and not limited to B16 melanoma cells.

Recently, ovarian G receptor-1, a G protein-coupled recep-tor-1, has been identified as a proton receptor, or sensor for acidic pH*e*, in osteoblasts (51). We tested the involvement of this protein in our system using pertussis and SCH-202676 (*N*-(2,3-diphenyl-l,2, 4-thiadiazol-5-(2H)-ylidene) methanamine), an inhibitor of both agonist and antagonist binding to G protein-coupled receptors. However, neither pertussis nor SCH-202676 reduced acidic pH*e*-induced MMP-9 expression. Moreover, we could not detect ovarian G receptor-1 mRNA expression by RT-PCR. These results suggest that the involvement of ovarian G receptor-1 or related receptors is unlikely in the acidic pH*e* induction of MMP-9.

In conclusion, we have shown here that the PLD-MAPKs ERK1/2 and p38 and NFκB activation are essential for the induction of MMP-9 expression at acidic pH*e*. Our findings may provide new clues to the mechanism of tumor invasion and metastasis and, therefore, on their prevention.

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REFERENCES

1. Bond, M., Fabunmi, R. P., Baker, A. H., and Newby, A. C. (1998) *FEBS Lett.* 435, 29-34

2. Wakelam, M. J., Martin, A, Hodgkin, M. N., Brown, F., Pettitt, T. R., Cross, M. J., De Takats, P. G., and Reynolds, J. L. (1997) *Adv. Enzyme Regul.* 37, 29-34

3. Williger, B. T., Ho, W. T., and Exton, J. H. (1999) *J. Biol. Chem.* 274, 735-738

4. Simon, C, Goepfert, H., and Boyd, D. (1998) *Cancer Res.* 58, 1135-1139

5. Simon, C, Hicks, M. J., Nemechek, A. J., Mehta, R., O'Malley, B. W., Jr.. Goepfert, H, Flaitz, C. M., and Boyd, D. (1999)*Br. J. Cancer* 80, 1412-1419

6. Shin, M., Yan, C, and Boyd, D. (2002) *Biochim. Biophys. Acta* 1589, 311-316

7. Kozin, S. V., Shkarin, P., and Gerweck, L. E. (2001) *Cancer Res.* 61, 4740-4743

8. Helmlinger, G., Sckell, A., Dellian, M., Forbes, N. S., and Jain, R. K. (2002) *Clin. Cancer Res.* 8, 1284-1291

9. Rotin, D., Robinson, B., and Tannock, I. F. (1986) *Cancer Res.* 46, 2821-2826

10. Tannock, I. F., and Rotin, D. (1989) *Cancer Res.* 49, 4373-4384

11. Yamagata, M., and Tannock, I. F. (1996) Br. *J. Cancer* 73, 1328-1334

12. Vukovic, V., and Tannock, I. F. (1997) *Br. J. Cancer* 75, 1167-1172

13. Yamagata, M., Hasuda, K, Stamato, T., and Tannock, I. F. (1998) *Br. J. Cancer* 77, 1726-1731

14. Kato, Y., Nakayama, Y., Umeda, M., and Miyazaki, K. (1992) *J. Biol. Chem.* 267, 11424-11430

15. Griffiths, L., Dachs, G. U., Bicknell, R., Harris, A. L., and Stratford, I. J. (1997) *Cancer Res.* 57, 570-572

16. Bellocq, A., Suberville, S., Philippe, C, Bertrand, F., Perez, J., Fouqueray, B., Cherqui, G., and Baud, L. (1998) *J. Biol. Chem.* 273, 5086-5092

17. Fukumura, D., Xu, L., Chen, Y., Gohongi, T., Seed, B., and Jain, R. K. (2001) *Cancer Res.* 61, 6020-6024

18. Xu, L., Fukumura, D., and Jain, R. K. (2002) *J. Biol. Chem.* 277, 11368-11374

19. Shi, Q., Abbruzzese, J. L., Huang, S., Fidler, I. J., Xiong, Q., and Xie, K. (1999) *Clin. Cancer Res.* 5, 3711-3721

20. Shi, Q., Le, X., Wang, B., Xiong, Q., Abbruzzese, J. L., and Xie, K. (2000) *J. Interferon Cytokine Res.* 20, 1023-1028

21. Xiong, Q., Shi, Q., Le, X., Wang, B., and Xie, K. (2001) *J. Interferon Cytokine Res.* 21, 529-537

22. Xu, L., and Fidler, I. J. (2000) *Cancer Res.* 60, 4610-4616

23. Sato, H., and Seiki, M. (1993) *Oncogene* 8, 395-405

24. Gum, R., Wang, H., Lengyel, E., Juarez, J., and Boyd, D. (1997) *Oncogene* 14, 1481-1493

25. Esteve, P. O., Chicoine, E., Robledo, O., Aoudjit, F., Descoteaux, A., Potworowski, E. F., and St-Pierre, Y. (2002) *J. Biol. Chem.* 277, 35150-35155

26. Kato, Y., Ozono, S., Shuin, T., and Miyazaki, K. (1996) *Cell Biol. Int.* 20, 375-377

27. Caumont, A. S., Galas, M. C, Vitale, N, Aunis, D., and Bader, M. F. (1998) *J. Biol. Chem.* 273, 1373-1379

28. Le Stunff, H., Dokhac, L., Bourgoin, S., Bader, M. F., and Harbon, S. (2000) *Biochem. J.* 3522, 491-499

29. Kato, Y., Lewalle, J. M., Baba, Y., Tsukuda, M., Sakai, N, Baba, M., Kobayashi, K., Koshika, S., Nagashima, Y., Frankenne, F., Noel, A., Foidart, J. M., and Hata, R. I. (2001) *Biochem. Biophys. Res. Commun.* 287, 422-426

30. Labarca, C, and Paigen, K. (1980) *Anal. Biochem.* 102, 344-352

31. Kato, Y-, Nagashima, Y., Koshikawa, N, Miyagi, Y., Yasumitsu, H., and Miyazaki, K. (1998) *Eur. J. Cancer* 34, 1117-1123

32. Baba, Y., Tsukuda, M., Mochimatsu, I., Furukawa, S., Kagata, H., Yoji, Nagashima, Sakai, N., Koshika, S., Imoto, M., and Kato, Y. (2000) *Clin. Exp. Metastasis* 18, 273-279

33. Vogt, A, Pestell, K. E., Day, B. W., Lazo, J. S., and Wipf, P. (2002)*Mol. Cancer Ther.* 1, 885-892

34. Zamorano, J., Rivas, M. D., Garcia-Trinidad, A., Qu, C. K, and Keegan, A. D. (2003) *J. Immunol.* 171, 4203-4209

35. Kato, Y., Ozono, S., and Koshika, S. (1996) *J. Steroid Biochem. Mol. Biol.* 57, 349-355

36. Banno, Y., Fujita, H., Ono, Y., Nakashima, S., Ito, Y., Kuzumaki, N., and Nozawa, Y. (1999) *J. Biol. Chem.* 274, 27385-27391

37. Lambert, C. A., Colige, A. C, Munaut, C, Lapiere, C. M., and Nusgens, B. V. (2001) *Matrix Biol.* 20, 397-408

38. Hu, Q., Natarajan, V., and Ziegelstein, R. C. (2002) *Biochem. Biophys. Res. Commun.* 292, 325-332

39. Moon, Y., and Pestka, J. J. (2002) *Toxicol. Sci.* 69, 373-382

40. Attiga, F. A., Fernandez, P. M., Weeraratna, A. T., Manyak, M. J., and Patierno, S. R. (2000) *Cancer Res.* 60, 4629-4637

41. Lakka, S. S., Jasti, S. L., Gondi, C, Boyd, D., Chandrasekar, N., Dinh, D. H., Olivero, W. C, Gujrati, M., and Rao, J. S. (2002) *Oncogene* 21, 5601-5608

42. Hong, J. H., Oh, S. O., Lee, M., Kim, Y. R., Kim, D. U., Hur, G. M., Lee, J. H., Lim, K, Hwang, B. D., and Park, S. K. (2001) *Biochem. Biophys. Res. Commun.* 281, 1337-1342

43. Bechoua, S., and Daniel, L. W. (2001) *J. Biol. Chem.* 276, 31752-31759

44. Eberhardt, W., Schulze, M., Engels, C, Klasmeier, E., and Pfeilschifter, J. (2002) *Mol. Endocrinol.* 16, 1752-1766

45. Himelstein, B. P., Lee, E. J., Sato, H, Seiki, M., and Muschel, R. J. (1998) *Clin. Exp. Metastasis* 16, 169-177

46. Roebuck, K. A., Carpenter, L. R., Lakshminarayanan, V., Page, S. M., Moy, J. N, and Thomas, L. L. (1999) *J. Leukocyte Biol.* 65, 291-298

47. Cummings, R. J., Parinandi, N. L., Zaiman, A., Wang, L., Usatyuk, P. V., Garcia, J. G., and Natarajan, V. (2002) *J. Biol. Chem.* 277, 30227-30235

48. Hwang, Y. S., Jeong, M., Park, J. S., Kim, M. H, Lee, D. B., Shin, B. A, Mukaida, N, Ellis, L. M., Kim, H. R., Ahn, B. W., and Jung, Y. D. (2004) *Oncogene* 23, 6603-6611

49. Tanaka, T., Kanai, H, Sekiguchi, K, Ahara, Y., Yokoyama, T., Arai, M., Kanda, T., Nagai, R., and Kurabayashi, M. (2000) *J. Mol. Cell. Cardiol.* 32, 1955-1967

50. Ieda, Y., Waguri-Nagaya, Y., Iwahasi, T., Otsuka, T., Matsui, N., Namba, M., Asai, K, and Kato, T. (2001) *Rheumatol. Int.* 21, 45-52

51. Ludwig, M. G., Vanek, M., Guerini, D., Gasser, J. A., Jones, C. E., Junker, U., Hofstetter, H, Wolf, R. M., and Seuwen, K. (2003) *Nature* 425, 93-98