Localization of a cyclopentenone prostaglandin to the endoplasmic reticulum and induction of BiP mRNA

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Cyclopentenone prostaglandins (PGs) are transported into cells and stimulate the expression of various stress genes, such as that coding for BiP (an ER luminal protein). To reveal the site of action of the PGs for the induction of stress-gene expression, we introduced a fluorescent probe, pyrene, into two types of PG analogue, GIF0010 (a cyclopentenone type) and GIF0037 (a cyclopentanone type) and examined their intracellular localization in normal rat kidney cells and their ability to induce the BiP gene expression. GIF0010 accumulated around the nuclei and coincided with BiP, a resident protein in the endoplasmic reticulum (ER) and markedly induced BiP gene expression. By contrast, GIF0037 and pyrene neither accumulated in the cell nor induced BiP gene expression. Thus the ER localization of GIF0010 and the induction of gene expression by GIF0010 are ascribed to the cyclopentenone structure. Treatment with cycloheximide inhibited both the accumulation of GIF0010 and the induction of the BiP mRNA, suggesting that the ER localization of the PG and subsequent gene expression require the nascent protein synthesis. These results demonstrate that the cyclopentenone PG is specifically accumulated in the ER, transducing a signal for BiP gene expression in the nuclei.

INTRODUCTION

Prostaglandins (PGs) are oxygenated metabolites of arachidonic acid and are regarded as modulators of cellular function in various physiological and pathological processes [1]. Conventional PGs such as PGE_2 and PGD_2 act on a cell surface receptor to exert their actions, and the molecular structures of their receptors have recently been revealed [2]. In contrast with PGs acting on a cell-surface receptor, cyclopentenone PGs, such as Δ^{12}-PGJ_2 and PGA_2, have no cell-surface receptor, but are actively transported into cells and exert a variety of biological actions, including the inhibition of cell-cycle progression, suppression of viral replication, cell differentiation and development and stimulation of osteogenesis [3,4].

Among them, the prominent action of cyclopentenone PGs is a stress response. Cyclopentenone PGs are produced in response to various stress stimuli, and are then actively transported into cells and induce the expression of various stress-related protein genes, including those of a family of cytosolic heat-shock proteins [5,6], ribosome-inactivating protein [7], haem oxygenase [8], protein disulphide-isomerase [9] and BiP (an ER luminal protein) [10].

Narumiya et al. reported that Δ^{12}-PGJ_2 is actively transported into cells by a specific carrier and accumulates in the nuclear fraction [11–13]. Since this uptake and accumulation were closely correlated with the growth-inhibitory activity of cyclopentenone PGs, the primary target(s) of this type of PG has been thought to be in the nuclei. However, the PG-induced stress-protein-gene expression has been shown to require *de novo* protein synthesis [6,7,10]. The requirement of *de novo* protein synthesis for the cyclopentenone PG-induced gene expression suggests that the site of stress actions of the PGs may be on the regulation of nascent protein processing. We have recently confirmed that Δ^{12}-PGJ_2 induced the expression of BiP gene through an unfolded protein response element (UPRE) that responds to the accumulation of unfolded proteins in the endoplasmic reticulum (ER) [10], suggesting that the stress actions of cyclopentenone PGs are correlated with an unfolded protein response and the primary site of action of the PGs is not on nuclei. Thus determination of the target(s) of cyclopentenone PGs is urgently required if one is to elucidate the molecular mechanisms of the stress-gene expression.

In the present study we prepared two types of PG analogue with a fluorescent probe, a cyclopentenone type and a cyclopentanone type, and investigated their intracellular localization and the effects on Bip gene expression. We report here that the ER is a target of the cyclopentenone PG and the accumulation in the ER is a trigger to induce Bip gene expression.

EXPERIMENTAL

Materials

Δ^{12}-PGJ_2 and a monoclonal antibody against rat TGN38 (a *trans*-Golgi-network membrane protein; clone 2F7.1) were generously given by Dr. Kurozumi of Teijin Ltd. (Tokyo, Japan) and Dr. George Banting of the Department of Biochemistry, University of Bristol, Bristol, U.K., respectively. Agents obtained and commercial sources were as follows: [α-^{32}P]dCTP

Abbreviations used: PG, prostaglandin; UPRE, unfolded protein response element; ER, endoplasmic reticulum; Man II, mannosidase II; NRK, normal rat kidney; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; PPAR, peroxisome proliferator-activated receptors.

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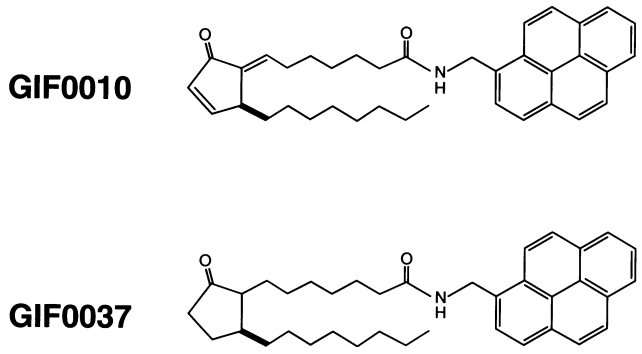

Figure 1 Chemical structures of GIF0010 and GIF0037

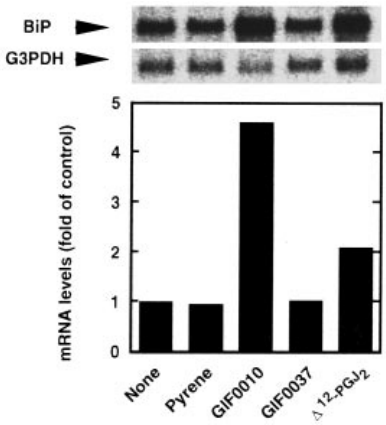

Figure 2 Effects of fluorescent probe-labelled PGs on the BiP mRNA level in NRK cells

After NRK cells (5×10^5 cells) had been treated with vehicle ('None'), 10 μM pyrene or 10 μM indicated PG for 12 h, total cellular RNA was extracted and subjected to Northern-blot analysis, as described in the Experimental section. Arrows indicate the hybridized bands for BiP and the G3PDH mRNA. The values of BiP mRNA are normalized to that of the G3PDH mRNA and expressed as fold increase over control. The results are representative of three independent experiments that yielded similar results.

(3000 Ci/mmol), Du Pont–New England Nuclear (Boston, MA, U.S.A.); a monoclonal antibody against rat mannosidase II (Man II, an integral Golgi membrane protein; clone 53FC3), Berkeley Antibody Company (Richmond, CA, U.S.A.); rabbit polyclonal antisera against BiP, StressGen Biotechnologies Corp. (Victoria, BC, Canada); a rhodamine-labelled goat antibodies to mouse IgG and to rabbit IgG, Leinco Technologies Inc. (St. Louis, MO, U.S.A.); a rhodamine-labelled goat antibody to rat IgG, Kerkegaard & Perry Laboratories Inc. (Gaithersburg, MD, U.S.A.); and pyrene and cycloheximide, Nakalai tesque (Kyoto, Japan). The sources of other materials are given in the text. Normal rat kidney (NRK) cells were grown in high-glucose (11 mM) Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal-bovine serum and 4 mM glutamine under humidified air containing 5 % CO_2 at 37 °C.

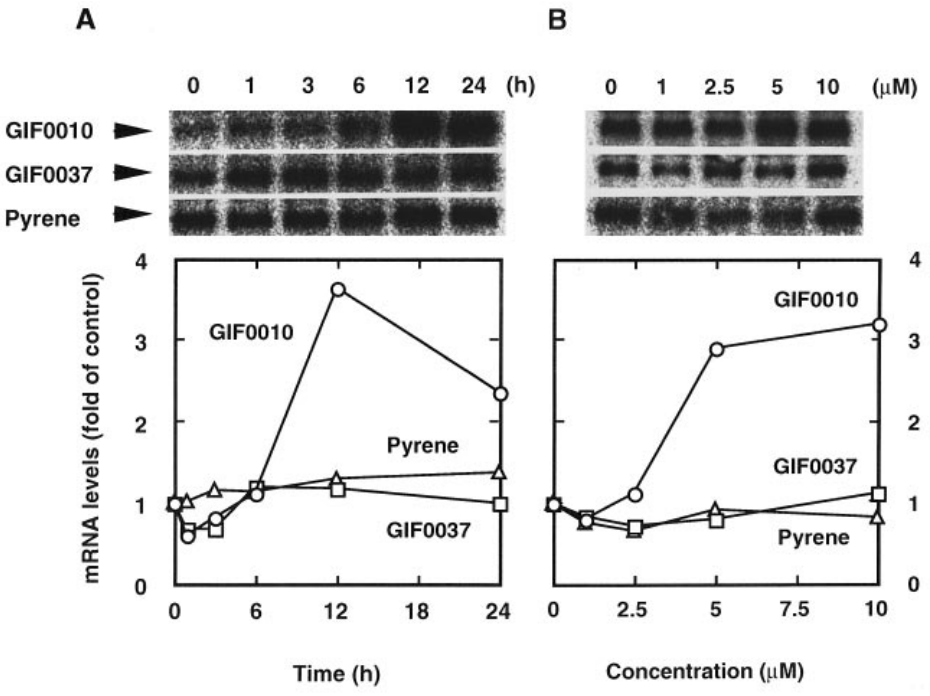

Figure 3 Time courses and concentration-dependencies of the effects of GIF0010, GIF0037 and pyrene on BiP mRNA level

(**A**) Time course. After the cells (5×10^5 cells) had been treated with 10 μM GIF0010 (○), GIF0037 (□) or pyrene (△) for the indicated times, total cellular RNA was extracted from the cells. (**B**) Concentration-dependency. After the cells (5×10^5 cells) had been treated with the indicated concentrations of GIF0010 (○), GIF0037 (□) or pyrene (△) for 12 h, total cellular RNA was extracted. Total cellular RNA (5 μg) was subjected to Northern-blot analysis as described in the Experimental section. Arrows indicate the hybridized bands for BiP mRNA. The values of BiP mRNA are normalized to that of the G3PDH mRNA and expressed as fold increase over the untreated-cell value. The results are representative of three independent experiments that yielded similar results.

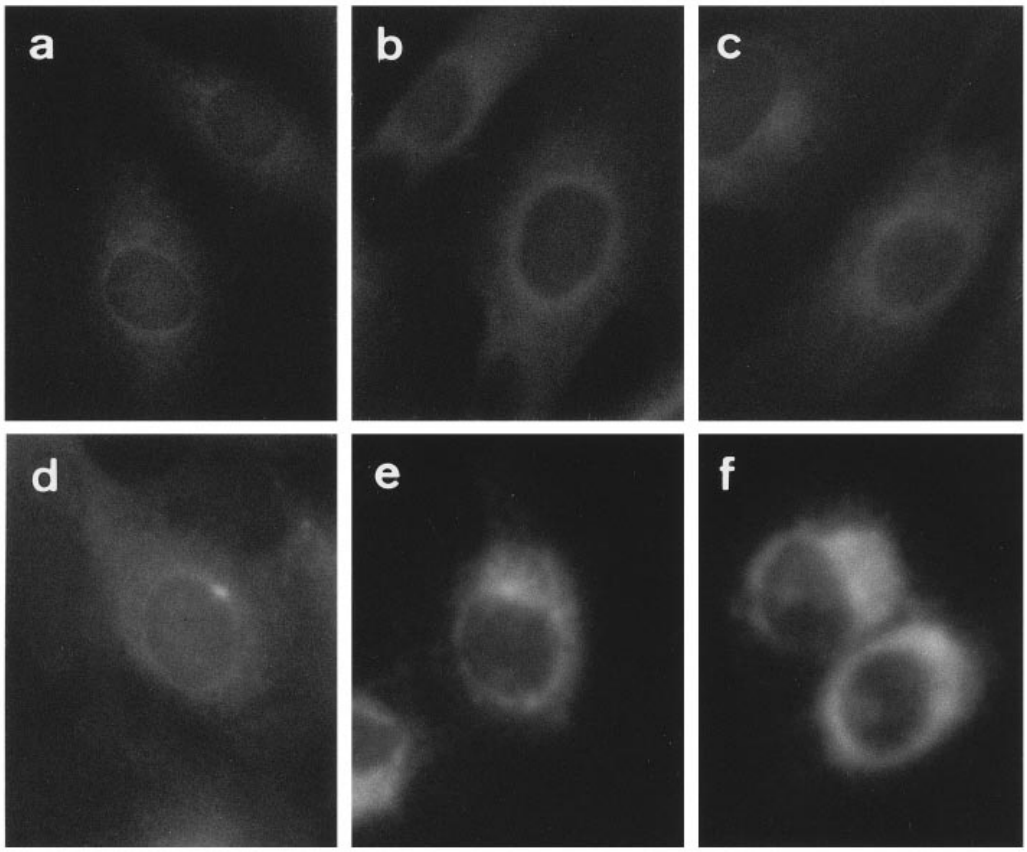

Figure 4 Time course of the intracellular localization of GIF0010

After the cells (4×10^4) had been treated with 10 μM of GIF0010 for 0 h (**a**), 1 h (**b**), 3 h (**c**), 6 h (**d**), 12 h (**e**) or 24 h (**f**), the cells were fixed and analysed by fluorescence microscopy through a UV filter as described in the Experimental section. The results are representative of three independent experiments that yielded similar results.

Northern blotting

Total RNA from NRK cells was isolated using an Isogen RNA isolation kit (Nippon-gene, Tokyo, Japan), and 5 μg of each RNA was separated by electrophoresis on a 1.5 %-agarose gel, transferred to a membrane (GeneScreen Plus; Du Pont–New England Nuclear) and hybridized with a ^{32}P-labelled *Bam*HI/*Sal*I fragment of the plasmid pSV-BiP [14]. The same filter was rehybridized with ^{32}P-labelled glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA probe (Clontech, Palo Alto, CA). Hybridization was carried out at 65 °C in 6 × SSC, and the filter was washed at 65 °C in 2 × SSC. Radioactivities of the bands were determined using a BAS 2000 bioimaging analyser (Fuji Photo Film Co., Tokyo, Japan).

Indirect immunofluorescence microscopy

NRK cells were cultured on coverglasses (diameter 18 mm) for 1 day. After treatment with the fluorescent PGs, the cells were washed twice with PBS and fixed with methanol (5 min, −20 °C). On that occasion we chose methanol fixation because it had been shown to be more suitable than aldehyde fixation for the immunofluorescence analysis using the monoclonal antibody to TGN38 [15]. On the other hand, with regard to the fluorescent PGs, methanol and paraformaldehyde fixations showed the same staining patterns. Fluorescent PGs gave a fluorescence with an emission peak at 395 nm and an excitation peak at 274 nm. For double staining for fluorescent PG and indirect immunofluorescence of several protein antibodies, the cells cultured on coverglasses were treated with PG for 6 h, rinsed with PBS twice and cultured for 12 h with PG-free fresh medium. The cells were fixed with methanol (5 min, −20 °C), rinsed with high-salt PBS (0.01 M sodium phosphate/0.5 M NaCl, pH 7.3) and blocked with PBS containing 2 % fetal-bovine serum (30 min, 20 °C). The cells were then incubated sequentially with a primary antibody (1 h, 4 °C) and a rhodamine-labelled anti-mouse, anti-rat or anti-rabbit IgG (1 h, 20 °C). Excess secondary antibody was thoroughly washed with high-salt PBS and the coverglasses were then mounted in 90 % glycerol containing 0.1 % *p*-phenylenediamine dihydrochloride (Sigma). The stained cells were examined by fluorescence microscopy using an Olympus model BX-FLA fluorescence microscope, and the original photographs were obtained with Ektachrome 400 film (Kodak). The fluorescence of PGs is more properly detected through a UV filter. To avoid the overlapping of fluorescence of PGs and the second antibody, we chose a rhodamine-labelled antibody as a second antibody. The photographs were taken at a magnification of × 400.

RESULTS

Effects of fluorescent PGs on the level of BiP mRNA

In order to reveal the intracellular localization of cyclopentenone PGs directly, we synthesized two fluorescent analogues, cyclo-

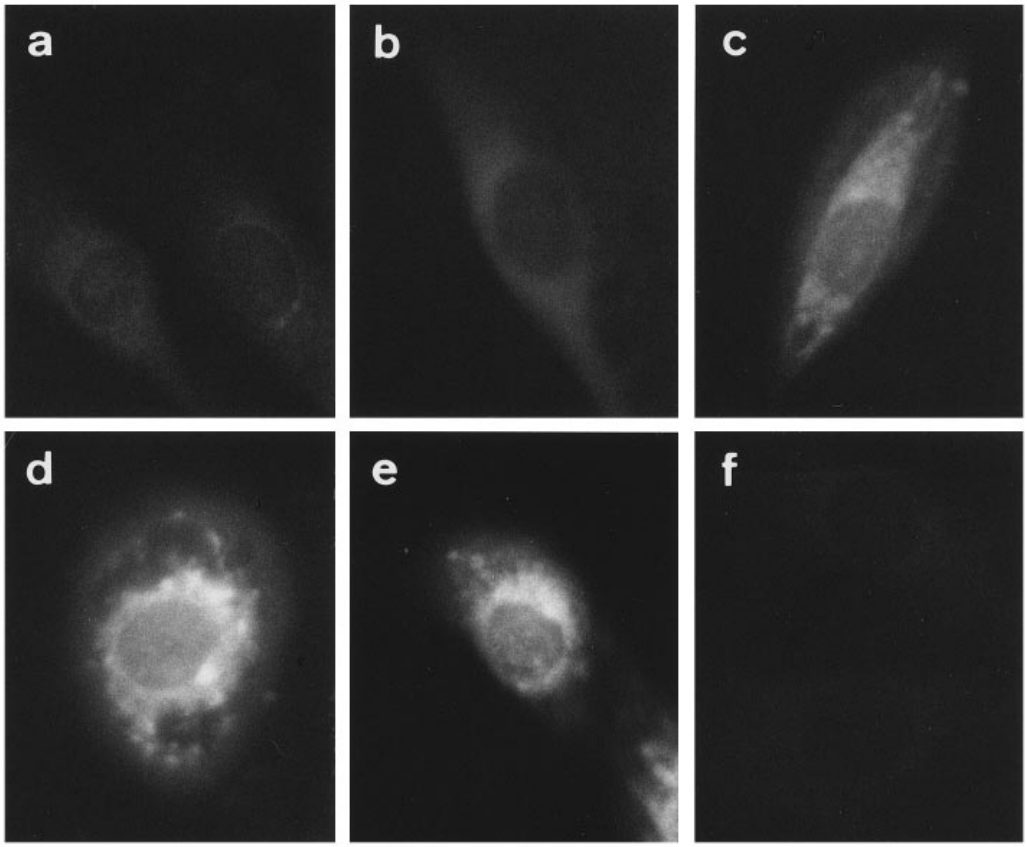

Figure 5 Concentration-dependency of the intracellular localization of GIF0010

After the cells (4×10^4) had been treated with vehicle (**a**), 1 μM (**b**), 2.5 μM (**c**), 5 μM (**d**), or 10 μM (**e**) of GIF0010 or 10 μM of GIF0037 (**f**) for 12 h, the cells were fixed and analysed by fluorescence microscopy through a UV filter as described in the Experimental section. The results are representative of three independent experiments that yielded similar results.

pentenone-type PG (GIF0010) and cyclopentanone-type PG (GIF0037) by conjugating a fluorescent probe, pyrene, with the corresponding acids prepared by three-component synthesis [16]. Figure 1 shows the chemical structures of both PGs. They differ only in the presence of a dienone in GIF0010, which has the ability to bind to thiol groups [16]; GIF0037 lacks the dienone structure.

We first examined the effects of GIF0010 and GIF0037 on the expression of the BiP gene in NRK cells by Northern-blot analysis. As shown in Figure 2, GIF0010 increased the mRNA level of BiP fourfold above the basal level, whereas GIF0037 caused no increase. Δ^{12}-PGJ$_2$ also increased the BiP mRNA level, but it was less than that of GIF0010, indicating that GIF0010 is a strongly potent inducer of BiP mRNA. On the other hand, pyrene did not increase the mRNA level of BiP.

We next examined the time courses and the concentration-dependencies of the effects of GIF0010, GIF0037 and pyrene on the BiP mRNA level. As shown in Figure 3(A), GIF0010 markedly increased the mRNA level of BiP to a maximum at 12 h. On the other hand, GIF0037 and pyrene did not increase it. The accumulation of BiP mRNA by GIF0010 was observed at 2.5 μM, and the level reached a maximum at 5 μM (Figure 3B). In contrast, GIF0037 and pyrene did not induce it up to 10 μM. The results indicate that GIF0010 is the potent inducer of BiP mRNA and the induction by GIF0010 is attributed to the cyclopentenone structure, but not to the pyrene structure.

Intracellular localization of fluorescent PGs

We next examined intracellular localization of the incorporated GIF0010 and GIF0037 in NRK cells under a fluorescence microscope. Figure 4 shows the time course of intracellular localization of GIF0010. The marked accumulation of GIF0010 was observed around the nucleus at 12 h. The nucleus was devoid of significant fluorescence. Figure 5 shows the concentration-dependency of the accumulation of GIF0010. Clear accumulation of fluorescence of GIF0010 around the nucleus was detected at 2.5 μM, and the intensity reached a plateau at 5 μM. The time course and the concentration-dependency of GIF0010 for the intracellular accumulation were quite consistent with those of GIF0010 for BiP mRNA induction. In contrast, significant accumulation of GIF0037 could not be observed (Figure 5f). Likewise, the fluorescence of pyrene was not detected in the cells after the incubation for 24 h (results not shown). These results indicate that the accumulation of GIF0010 around the nuclei can be ascribed to the cyclopentenone structure, but not to the pyrene structure.

The effects of Δ^{12}-PGJ$_2$, including cell-growth arrest, have been shown to be irreversible [13]. We then examined the reversibility of GIF0010-induced BiP gene expression and the intracellular accumulation. As shown in Figure 6(A), GIF0010 induced BiP mRNA continuously after the PG was removed from the medium. The accumulation of GIF0010 proceeded after the following incubation in the PG-free medium (Figure 6B,

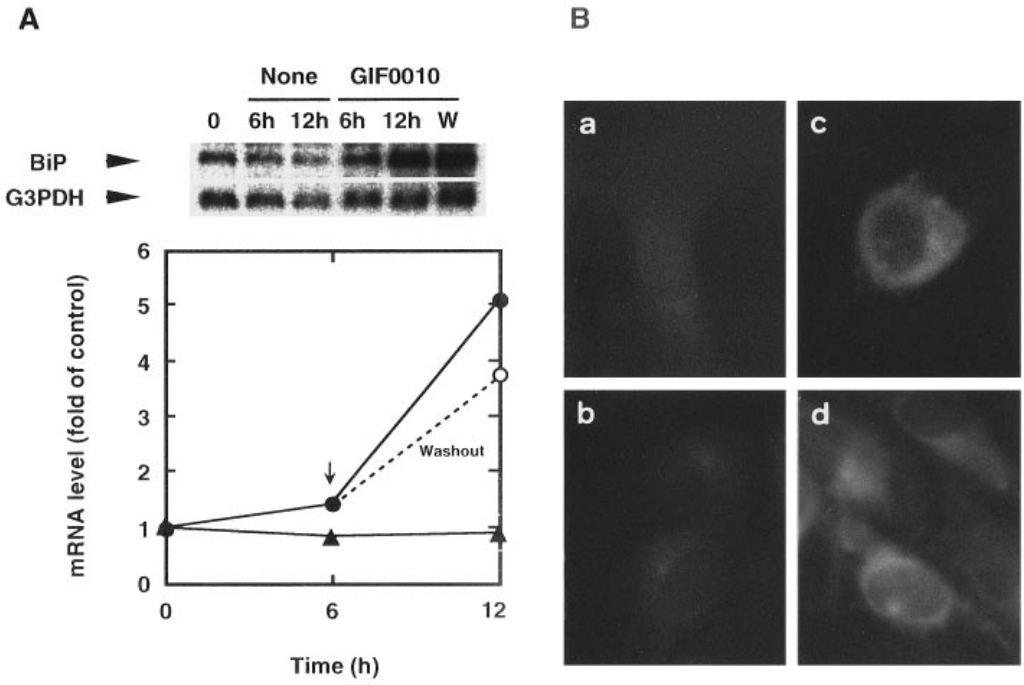

Figure 6 Reversibility of GIF0010-induced BiP gene expression and the intracellular localization of GIF0010

(**A**) BiP mRNA. After NRK cells (5×10^5) had been treated with vehicle (▲) or 10 μM of GIF0010 (●) for 6 h, they were washed with PBS twice at 6 h (arrow) and further cultured for 6 h in the conditioned medium in the presence (●) or absence [○, ▲ and (W)] of GIF0010. Total cellular RNA (5 μg) was extracted and subjected to Northern-blot analysis as described in the Experimental section. BiP mRNA levels were determined as described in the legend to Figure 3. (**B**) intracellular localization. After NRK cells (4×10^4) had been treated with vehicle (panel a) or 10 μM of GIF0010 (panel b) for 6 h, they were washed with PBS twice and further cultured for 6 h in the conditioned medium in the presence (panel c) or absence (panel d) of 10 μM of GIF0010. The cells were fixed and then analysed by fluorescence microscopy through a UV filter as described in the Experimental section. The results are representative of three independent experiments that yielded similar results.

panel d). The irreversible accumulation of GIF0010 was closely correlated with the continuous induction of Bip gene expression.

To identify the site of the intracellular accumulation of GIF0010, we next performed indirect immunofluorescence with the antibodies against several proteins, namely Man II (an integral Golgi membrane protein), TGN38 (a *trans*-Golgi-network membrane protein) and BiP (an ER luminal protein), and we compared fluorescent patterns of GIF0010 and the respective proteins. As shown in Figure 7, the fluorescence of GIF0010 was completely coincident with the stain of BiP and clearly different from that of Man II or TGN38, indicating that GIF0010 was specifically localized at the ER.

Effects of cycloheximide on GIF0010-induced BiP gene expression and ER localization

The BiP gene has been shown to be induced by the accumulation of improperly folded proteins newly synthesized in the ER [17] and we have recently shown that *de novo* protein synthesis is required for Δ^{12}-PGJ_2-induced expression of BiP gene [10]. We then investigated whether the GIF0010-induced expression of BiP gene and the ER accumulation were dependent on *de novo* protein synthesis. As shown in Figure 8(A), cycloheximide treatment completely abolished the GIF0010- as well as Δ^{12}-PGJ_2-induced accumulation of BiP mRNA, suggesting that *de novo* protein synthesis is required for the GIF0010-induced expression of BiP gene. Cycloheximide treatment also strongly suppressed the ER localization of GIF0010 (Figure 8B), suggesting that newly synthesized proteins participate in the ER localization of GIF0010.

DISCUSSION

Cyclopentenone PGs are actively transported into cells and induce the expression of various stress-related protein genes [5–10]. However, little is known about the site of action of PGs. In the present study we first discovered that cyclopentenone PG is accumulated in the ER and transmits the signal for the stress-gene expression in the nuclei, using novel PG analogues with a fluorescent probe.

Intracellular transport and distribution of cyclopentenone PGs have been analysed by biochemical approaches, such as subcellular fractionation of L-1210 murine leukemia cells, and the PGs were thought to have accumulated in the nuclei [11]. However, exact determination of the intracellular localization of the PGs cannot be accomplished by such biochemical approaches, because artificial redistribution of the PGs may occur during fractionation of cell homogenates, and clear separation of intracellular organelles and nuclei is very difficult. To resolve these drawbacks, we developed novel analogues of PG with a fluorescent probe, pyrene, to 'chase' the intracellular localization of the cyclopentenone PG. In the present study we revealed that the fluorescent cyclopentenone PG GIF0010 was accumulated in the ER, but not in the nuclei, in NRK cells and induced BiP gene expression (Figures 2 and 7). In contrast with GIF0010, neither

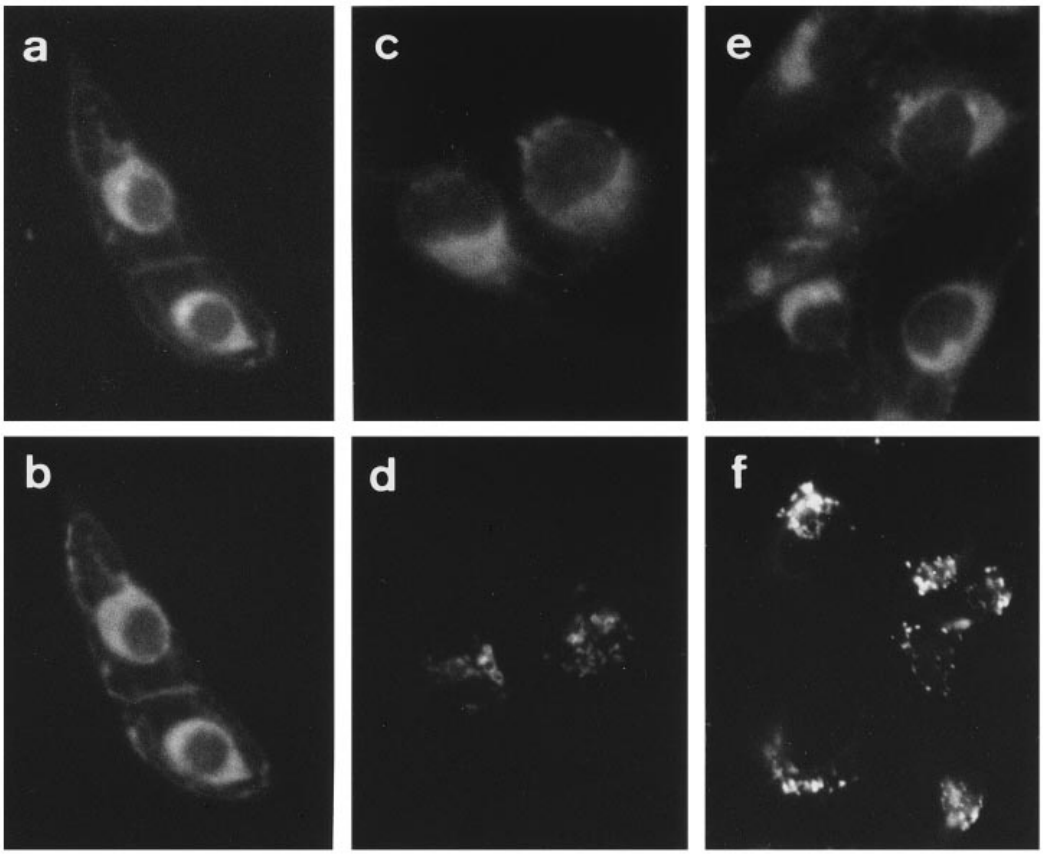

Figure 7 Co-localization of GIF0010 with BiP

After NRK cells had been incubated with 10 μM GIF0010 for 6 h, the cells were rinsed with PBS twice and further incubated for 12 h in the absence of GIF0010. The cells were then fixed and subjected to immunofluorescence analysis as described in the Experimental section. Photographs show cells double-stained with GIF0010 (**a**) and anti-BiP (**b**), GIF0010 (**c**) and anti-Man II (**d**), GIF0010 (**e**) and anti-TGN38 (**f**). The results are representative of three independent experiments that yielded similar results.

the fluorescent cyclopentanone PG GIF0037 nor pyrene showed ER accumulation, nor did they induce gene expression (Figures 2 and 5f). These results indicate that the ER accumulation of the cyclopentenone PG is linked to BiP gene expression and the linkage is ascribed to the cyclopentenone structure of GIF0010. We could not observe clear fluorescent signals of GIF0037, a cyclopentanone-type PG, in NRK cells. Although we cannot exclude the possibility that GIF0037 is not transported through the plasma membrane, the localization of GIF0037 in the cytoplasm may be marginal in the detection of PG fluorescence or this may be due to a great loss of the cytoplasmic PG throughout cell fixation.

The binding of the fluorescent cyclopentenone PG to the ER was irreversible (Figure 6B). We also showed that the induction of BiP mRNA by GIF0010 proceeded continuously after the PG was removed from the medium (Figure 6A). Thus this continuous induction of BiP gene expression is quite consistent with the irreversible association of the PG with the ER. Cyclopentenone PGs such as Δ^{12}-PGJ$_2$ and GIF0010, possessing a cross-conjugated dienone unit, react specifically with thiol nucleophiles and form irreversible and stable adducts with thiols of macromolecules such as protein thiols [16,18,19]. By contrast, GIF0037, a cyclopentanone PG without the dienone structure, cannot interact with thiols. The irreversibility of binding of the dienone PGs to thiols is correlated with their biological activities, such as irreversible anti-tumour activity [18]. With regard to the chemical and biological properties of dienone PGs, irreversible binding of the PG to thiol group(s) of a target molecule(s) in the ER may be a trigger for the induction of BiP gene expression.

How do the cyclopentenone PGs transduce the signal for the induction of BiP gene expression from the ER to the nucleus? It is now known that BiP gene expression is induced by various ER stresses, such as glucose starvation, inhibition of glycosylation with tunicamycin, disturbance of ER Ca^{2+}, treatment with amino acid analogues and exposure to heavy metals, which are thought to induce accumulation of unfolded proteins in the ER, and these ER stresses induce the gene expression through UPRE [20–22]. We previously revealed that the cyclopentenone PG-induced BiP gene expression was mediated by UPRE [10]. Thus cyclopentenone PGs and the abovementioned ER stress inducers use the same *cis*-regulatory element, UPRE, for BiP gene expression. Molecular mechanisms of ER stress signals to BiP gene expression has been extensively studied in yeast [22]. Recently, the transmembrane kinase Ire1p has been found to be localized in the ER or inner nuclear membrane and seems to be required for the induction of BiP gene through the activation of UPRE in yeast by the ER stresses [23–25], and the UPRE response was shown to be triggered by the oligomerization and the subsequent phosphorylation of Ire1p [26,27]. In addition, Hac1p, a transcription factor for UPRE, was cloned from yeast and shown to play a critical role in BiP gene expression in response to the ER stresses [28–30]. Furthermore, Li et al. have recently demon-

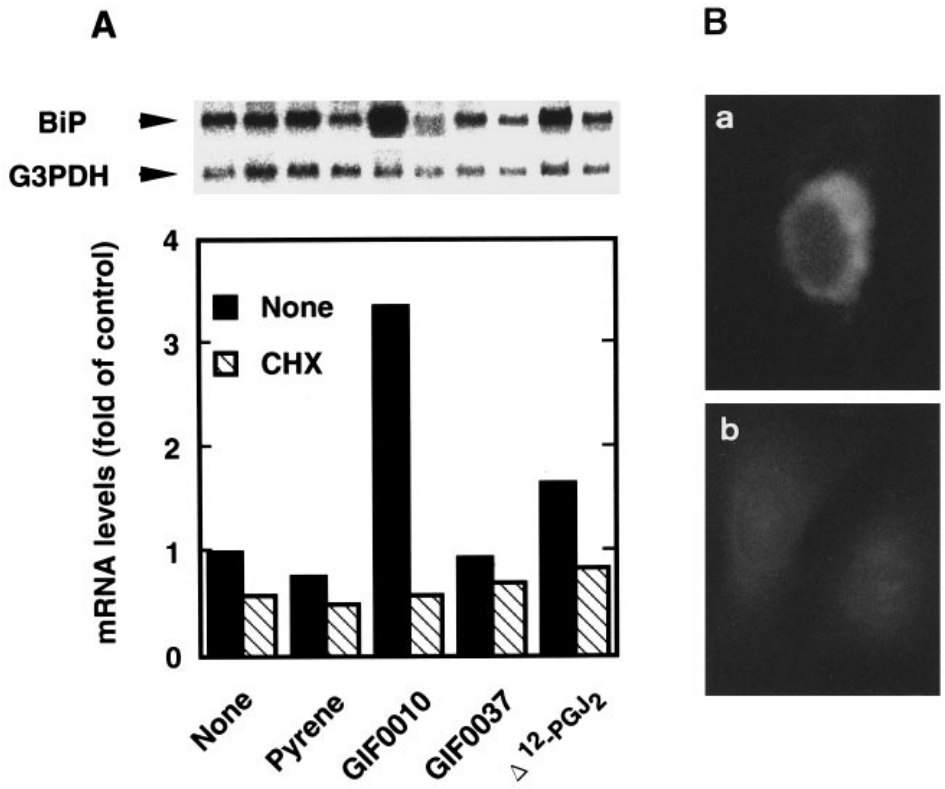

Figure 8 Effects of cycloheximide on the intracellular localization of GIF0010 and Bip gene expression

(**A**) After NRK cells (5×10^5) had been incubated with the vehicle (solid bars) or 1 μg/ml cycloheximide (hatched bars) for 30 min, they were further incubated with the vehicle ('None'), 10 μM of pyrene, GIF0010, GIF0037 or Δ^{12}-PGJ_2 for 12 h. Total cellular RNA was extracted and subjected to Northern-blot analysis as described in the Experimental section. Bip mRNA levels were determined as described in the legend to Figure 3. (**B**) after the cells had been incubated with the vehicle (panel a) or 1 μg/ml cycloheximide (panel b) for 30 min, they were further incubated with 10 μM GIF0010 for 12 h. The cells were then fixed and analysed by fluorescence microscopy through a UV filter as described in the Experimental section. The results are representative of three independent experiments that yielded similar results.

strated that the human nuclear transcription factor, YY-1, bound to core promoter element within UPRE and enhanced the transcription activation of BiP gene promoter in HeLa cells [31]. Thus nuclear transcription factors for UPRE have been well characterized in both yeast and mammalian cells, but the pathways for signals from the ER stresses to the nuclear factors remain unclear. In the present study we showed that the ER accumulation of the PG and the induction of BiP gene expression were sensitive to cycloheximide (Figures 8A and B), suggesting that the ER localization of the PG and the gene expression require the nascent protein synthesis. Most secretory and membrane proteins are synthesized on the ribosomes and transferred into the lumen of the ER. The nascent proteins have thiols in the molecules before they are folded and stabilized by disulphide-bond formation. Considering that cyclopentenone PGs are highly reactive with thiol groups, the target of cyclopentenone PGs in the ER may be thiols of nascent proteins. Moreover, it has been recently reported that accumulation of unfolded or misfolded proteins induced the formation of large aggregates which were associated with BiP in the ER [32,33]. It is therefore hypothesized that cyclopentenone PG binds to nascent proteins through their thiol groups to produce unfolded proteins in the ER. BiP recognizes the unfolded protein–cyclopentenone PG conjugate and forms aggregates, transducing stress signal(s) from the ER to the nuclei for induction of BiP gene expression.

Recently, 15-deoxy-$\Delta^{12,14}$-PGJ_2, the ultimate metabolite of PGD_2, has been identified to be a natural ligand of peroxisome proliferator-activated receptor γ (PPARγ), an orphan nuclear receptor, which is implicated in adipocyte differentiation and lipid homoeostasis [34,35]. This means that the cyclopentenone PG directly binds to the nuclear receptor and induces gene expression in adipocytes. However, the expression of PPARγ is specifically restricted in adipocytes [36]. On the other hand, the stress actions of cyclopentenone PGs can be observed ubiquitously [3]. The PPARγ activation and the stress responses induced by cyclopentenone PGs may be mediated by different mechanisms.

Our studies demonstrate that micromolar concentrations of cyclopentenone PG can be accumulated in the ER and induce BiP gene expression. PGD_2, a precursor of Δ^{12}-PGJ_2, is strongly synthesized by various cells, such as mast cells, during the process of inflammation, and this local concentration frequently reaches micromolar range [37]. Since PGD_2 is immediately converted into Δ^{12}-PGJ_2, the local concentrations of Δ^{12}-PGJ_2 would be elevated to the micromolar range. Although physiological concentrations of cyclopentenone PGs have not been established, previous studies have shown that cyclopentenone PGs mediate other activities at similar micromolar concentration range, such as cell-cycle arrest [38] and apoptosis [39]. The induction of BiP by the cyclopentenone PGs through their ER accumulation may play an important role in cytoprotective

regulation of protein folding under stress conditions. The present study will contribute not only to our understanding of the molecular mechanisms of cyclopentenone PG actions, but will also facilitate elucidation of mechanisms of the ER stress in gene expression.

This work was supported in part by Grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (09259219, 09273105 and 09307052) and by a grant from the Asahi Glass Research Foundation. S.T. was the recipient of a fellowship from the Japanese Society for the Promotion of Science for Japanese Junior Scientists. We thank Dr. S. Kurozumi, Dr. G. Banting and Dr. Y. Kozutsumi for supplying Δ^{12}-PGJ_2, a monoclonal antibody against rat TGN38 and pSV-BiP respectively.

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Received 22 May 1998/15 July 1998; accepted 4 August 1998