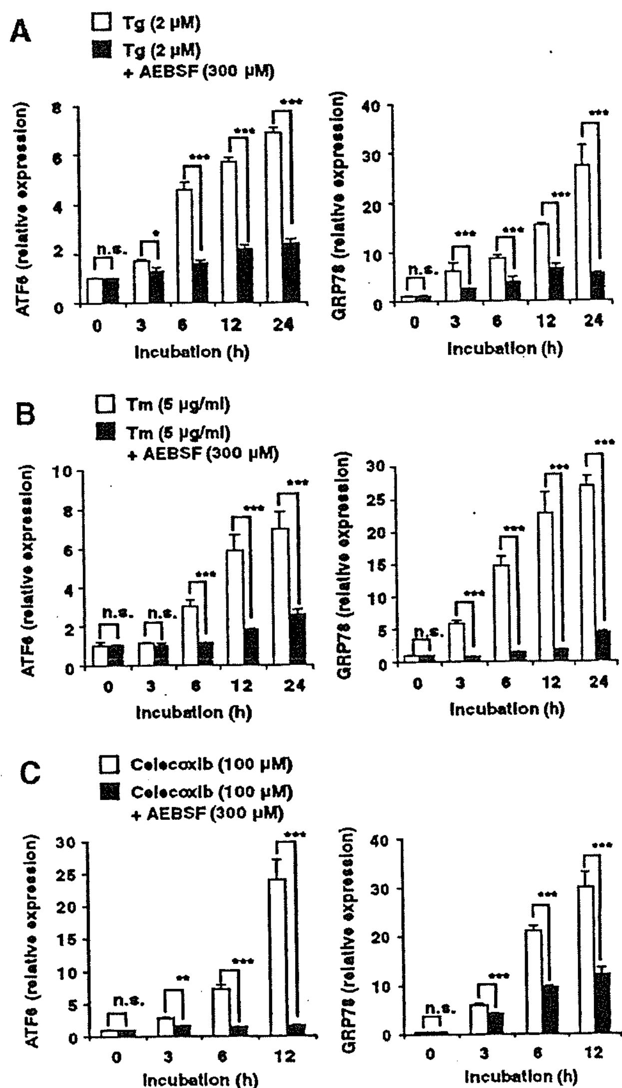

Fig. 3. E ect of AEBSF on ER stressor-induced up-regulation of *ATF6* mRNA expression. AGS cells were pre-incubated with or without 300 μM AEBSF for 1 h and then, still in the presence or absence of AEBSF, further incubated for the time periods indicated with thapsigargin (Tg) (A), tunicamycin (Tm) (B) or celecoxib (C). The levels of *ATF6* and *GRP78* mRNA were estimated by real-time RT-PCR experiments as described in the legend of Fig. 1. Values shown are means ± SD ($n = 3$). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. n.s. Not significant.

In order to examine the contribution of ATF6 to up-regulation of *ATF6* mRNA by ER stressors, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), an inhibitor of S1P, that was reported to inhibit the ER stressor-induced activation of ATF6 (degradation of p90-ATF6 into p50-ATF6) was used [16]. As shown in Fig. 3A, treatment of cells with AEBSF (300 μM) clearly inhibits the thapsigargin-dependent up-regulation of *ATF6* mRNA expression. We confirmed that thapsigargin-dependent up-regulation of *GRP78* mRNA expression (Fig. 3A) and the appearance of p50-ATF6 (data not shown) were inhibited by 300 μM AEBSF. Furthermore, this concentration of AEBSF did not decrease (but rather slightly increases) the amount of *ATF6* mRNA in the absence of thapsigargin (data not shown). Similar results were obtained with tunicamycin and celecoxib (Fig. 3B and C). AEBSF almost completely inhibited the celecoxib-dependent up-regulation of *ATF6* mRNA expression but only partially inhibited that of *GRP78* mRNA expression (Fig. 3C). This reflects the observation that ATF4 is involved in celecoxib-dependent up-regulation of *GRP78* mRNA expression [31] but not *ATF6* mRNA expression (Fig. 2F). In order to examine the specificity of this e ect of AEBSF, we examined its e ect on the celecoxib-induced up-regulation of *claudin 4* mRNA expression that was reported in our previous paper [5]. AEBSF did not inhibit (but rather slightly stimulated) celecoxib-induced up-regulation of *claudin 4* mRNA expression (data not shown), suggesting that the e ect of AEBSF is specific for *ATF6*. Overall, the results in Fig. 3 suggest that up-regulation of *ATF6* mRNA by ER stressors is mediated by activation of ATF6, in other words, by the S1P-dependent degradation of p90-ATF6 into p50-ATF6.

For confirmation of this idea, we examined the e ect of over-expression of the active form of ATF6 on the expression of ATF6. Transfection of cells with the plasmid pATF6(373), containing DNA sequences corresponding to amino acid residues 1–373 of ATF6, was reported to induce the ER stress response, suggesting that the translated fragment of ATF6 (ATF6(373)) acts as an active form of ATF6 similar to p50-ATF6 [10]. We confirmed that transfection with pATF6(373) caused up-regulation of not only ATF6(373) (data not shown) but also of *GRP78* mRNA and GRP78 protein (Fig. 4A and B). As shown in Fig. 4A, transfection of cells with pATF6(373) caused up-regulation of *ATF6* mRNA levels. Because the primers used in obtaining the results in Fig. 4A (for *ATF6*) do not recognize the mRNA derived from pATF6(373), the results in Fig. 4A show that over-expression of ATF6(373) caused up-regulation of expression of mRNA derived from the endogenous *ATF6*. Transfection with pATF6(373) also caused up-regulation of p90-ATF6 (Fig. 4B). The results in Fig. 4A and B suggest that the active form of ATF6 positively regulates the transcription of *ATF6*.

We searched for three types of ATF6-binding consensus sequences in the promoter of *ATF6* and found two ATF6-binding elements (TGACGT) (from 2525 to 2520 and from 987 to 982), based on consensus sequences described in a previous study [23]. Thus, it is possible that p50-ATF6 binds to these elements to induce the transcription of *ATF6* and that this induction is responsible for the up-regulation of *ATF6* mRNA expression by ER stressors.

A role for up-regulation of ATF6 mRNA expression in ER stress response

We assumed that the up-regulation of *ATF6* mRNA by ER stressors contributes to enhancement of the ER stress response; in other words, to enhancement of ER stressor-induced up-regulation of various genes such as *GRP78*. To test this idea, we examined the e ect of over-expression

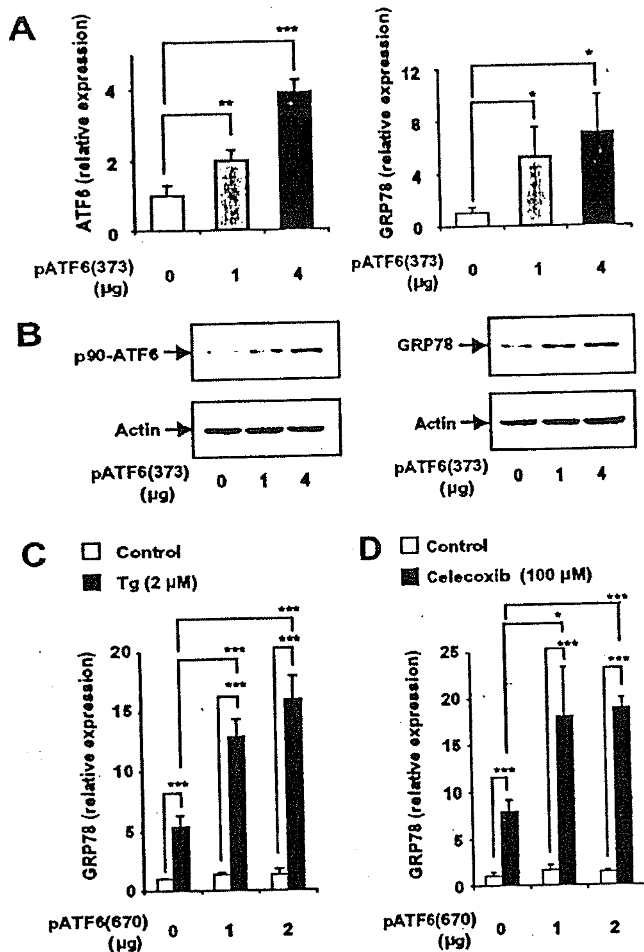

Fig. 4. Up-regulation of ATF6 and *ATF6* mRNA by the active form of ATF6 and e ect of over-expression of p90-ATF6 on the ER stress response. AGS cells were transiently transfected with the indicated amount of expression plasmid for ATF6(373) (pATF6(373)) and/or control vector (total DNA amounts were fixed at 4 μg) and cultured for 6 h (B) or 12 h (A) (A,B). AGS cells were transiently transfected with the indicated amount of expression plasmid for p90-ATF6 (pATF6(670)) and/or control vector (total DNA amounts were fixed at 2 μg) and cultured for 24 h. Cells were further incubated with or without the indicated concentration of thapsigargin (Tg) (C) or celecoxib (D) for 6 h (C,D). Whole cell extracts were analyzed by immuno-blotting with antibodies specific for p90-ATF6, GRP78 or actin (B). The levels of *ATF6* and *GRP78* mRNA were estimated by real-time RT-PCR experiments as described in the legend of Fig. 1. Values shown are means ± SD ($n = 3$). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ (A,C,D).

of p90-ATF6 on ER stressor-induced up-regulation of *GRP78* mRNA expression. Transfection of AGS cells with pATF6(670) (the expression plasmid for p90-ATF6) caused over-expression of p90-ATF6 and *ATF6* mRNA (data not shown). As shown in Fig. 4C and D, transfection with pATF6(670) stimulated the thapsigargin- or celecoxib-induced up-regulation of *GRP78* mRNA expression. For thapsigargin, similar results have previously been reported [21]. On the other hand, transfection with pATF6(670) did not increase the amount of *GRP78* mRNA in the absence of ER stressors (Fig. 4C and D). These results show that over-expression of p90-ATF6 cannot induce the ER stress response by itself; however, it can stimulate the ER stress response induced by ER stressors and suggests that up-regulation of *ATF6* mRNA expression by ER stressors contributes to enhancement of the ER stress response.

From the results of this study, we propose that various ER stressors induce the transcription of *ATF6* and that the active form of ATF6 (p50-ATF6) positively regulates the transcription of *ATF6*. As for the physiological role of this up-regulation, we have considered two possibilities. As described above, one possibility is that the up-regulation is involved in the stimulation of the ER stressor-induced ER stress response. The other possibility is that it is involved in the maintenance of p90-ATF6 in cells and in preparation for the next induction of the ER stress response. The amount of p90-ATF6 rapidly decreases upon exposure to ER stressors due to cleavage by S1P and S2P or proteasome-dependent degradation [16,32], however, it returns to the original level within 12–24 h [21]. This compensation may be necessary to ensure rapid induction of the next ER stress response and we assume that ER stressor-induced up-regulation of *ATF6* mRNA expression contributes to this compensation. It is also interesting to consider that maintenance of p90-ATF6 in cells may not only be important because it is the precursor for p50-ATF6 but because it may play other, as yet unknown, cellular roles.

Acknowledgments

We thank Dr. K. Mori (Kyoto University) for generously providing the plasmids used in this study. This work was supported by the Japan Science and Technology Agency.

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