Proteomic Identification of Bcl2-associated Athanogene 2 as a Novel MAPK-activated Protein Kinase 2 Substrate*

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The p38 MAPK cascade is activated by various stresses or cytokines. Downstream of p38 MAPKs, there are diversification and extensive branching of signaling pathways. Fluorescent two-dimensional difference gel electrophoresis of phosphoprotein-enriched samples from HeLa cells in which p38 MAPK activity was either suppressed or activated enabled us to detect ~90 candidate spots for factors involved in p38-dependent pathways. Among these candidates, here we identified four proteins including Bcl-2-associated athanogene 2 (BAG2) by peptide mass fingerprints. BAG family proteins are highly conserved throughout eukaryotes and regulate Hsc/Hsp70-mediated molecular chaperone activities and apoptosis. The results of two-dimensional immunoblots suggested that the phosphorylation of BAG2 was specifically controlled in a p38 MAPK-dependent manner. Furthermore, BAG2 was directly phosphorylated at serine 20 in vitro by MAPK-activated protein kinase 2 (MAPKAP kinase 2), which is known as a primary substrate of p38 MAPK and mediates several p38 MAPK-dependent processes. We confirmed that MAPKAP kinase 2 is also required for phosphorylation of BAG2 in vivo. Thus, p38 MAPK-MAPKAP kinase 2-BAG2 phosphorylation cascade may be a novel signaling pathway for response to extracellular stresses.

Mitogen-activated protein kinases (MAPKs) regulate a wide variety of cellular processes by phosphorylating their specific substrates. There are at least four distinctly regulated subgroups of MAPKs: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase 1/2 (JNK), and p38 mitogen-activated protein kinase 1/2 (p38 MAPK). ERK, c-Jun N-terminal kinase (JNK), and p38 MAPKs are highly conserved throughout eukaryotes and are a primary substrate of p38 MAPK and mediates several p38 MAPK-dependent processes. We confirmed that MAPKAP kinase 2 is also required for phosphorylation of BAG2 in vivo. Thus, p38 MAPK-MAPKAP kinase 2-BAG2 phosphorylation cascade may be a novel signaling pathway for response to extracellular stresses.

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§ The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MAPKAPK2, MAPK-activated protein kinase 2; siMAPKAPK2, short interfering MAPKAPK2; HA, hemagglutinin; 2D-DIGE, two-dimensional fluorescent difference gel electrophoresis; siRNA, short interfering RNA; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; JNK, c-Jun N-terminal kinase; pl, isoelectric point; BAG2, Bcl-2-associated athanogene 2; GST, glutathione S-transferase.
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Ran-binding protein as candidates for targets of p38 MAPK-dependent phosphorylation in response to anisomycin treatment in HeLa cells. Furthermore, we provided definitive evidence that MAPKAPK2 phosphorylates BAG2 at Ser^{20} in vitro and in vivo. These results demonstrate that BAG2 is a novel component of the p38 MAPK signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Materials**—HeLa and 293 cells were purchased from the ATCC (Manassas, VA). Both cell lines were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Transfections were performed using LipofectAMINE 2000 reagent (Invitrogen) following the manufacturer’s instructions. Protein kinase inhibitors for SB203580 for 50 min followed by treatment with 1 unit of ERK2 (active MAPK; Calbiochem) with 1.0 Ci of [γ-{32P}] in 20 μl of a kinase buffer (20 mM Tris, pH 7.5, 10 mM MgCl₂, 0.1 mM ATP), for 20 min at 30 °C. Reactions were terminated with Laemmli SDS sample buffer to a final volume of 30 μl, halves of samples were subjected to 10% SDS-PAGE, and phosphorylation reactions were visualized by autoradiography.

**Construction and Transfection of siMAPKAPK2**—A short interfering RNA (siRNA) targeting endogenous MAPKAPK2 was generated using BLOCK-iT Dicer RNAi kits (Invitrogen) following recommended protocols. Purified siMAPKAPK2 (250 or 500 ng/well) was used for transfection into 293 cells cultured in 6-well plates.

**Identification of Signaling Molecules in the p38 MAPK Cascade**—To globally identify factors involved in the p38 MAPK cascade, we developed a system consisting of phosphoprotein purification, fluorescent 2D-DIGE, and mass spectrometric identification of proteins. We prepared three lysates of HeLa cells (control untreated cells and cells treated with anisomycin in the presence or absence of SB203580), in which p38 MAPK activity was suppressed or activated, respectively. The specificity of SB203580 inhibition of p38 MAPK pathways was also confirmed as shown later. Phosphoproteins were enriched by using a commercially available purification kit. We confirmed that phosphorylated forms of p38 MAPK, MAPKAPK2, and HSP27 bound to the column and were eluted with relatively good recoveries from the column (data not shown).

Phosphoprotein-enriched fractions from the three lysates were labeled with cyanine dyes Cy3 and Cy5, respectively; combined, and run on the same gel. The Cy2 (control cells), Cy3 (anisomycin), and Cy5 (anisomycin + SB203580) signals were individually scanned at mutually exclusive excitation/emission wavelengths, which are exhibited as blue, red, and green pseudocolors (Fig. 1, A–C).

Fig. 1D shows a merge of three images. There were a large number of red spots, the amount of which was higher in p88-activated cells than in the other two cells (88 and 101 spots exhibiting a 1.5-fold or higher increase among 2865 and 2891 spots in two separate experiments employing different cell lysates). Since phosphoproteins were enriched before the analysis, these spots were candidates for proteins phosphorylated in p38 signaling pathways. Most of the remaining spots were white as a merge of blue, red, and green, demonstrating their equal quantities among three samples. These spots may be the phosphoproteins unrelated to the p38 pathway or proteins non-specifically trapped by the column. Green spots were also observed, some of which were characterized as described below.

**Determination of Proteins Using 2D-DIGE and Peptide Mass Fingerprinting**—Phosphoprotein-enriched sample derived from HeLa cells treated with anisomycin (650 μg, nonlabeled) was separated by two-dimensional gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and stained with Coomassie Blue. The spots corresponding to those of α-β in Fig. 1E (magnified view of a boxed region in D) were excised.
and subjected to peptide mass fingerprinting as described under “Experimental Procedures.” The MALDI-TOF mass spectrum of one of these proteins is shown in Fig. 2. Using the MASCOT data base search algorithms, this protein was identified as human BAG2 (spot a). Other spots were Hsp27 (spots b–d), human splicing factor arginine/serine-rich 9 (spot e), and human Ran-binding protein (spot f). Since the activation of p38 by anisomycin caused Hsp27 phosphorylation at Ser15, Ser78, and Ser 82 (21, 22), spots b–d may correspond to Hsp27 at different phosphorylation states. Ran-binding protein (spot f) exhibited a green spot suggesting a phosphorylation by a SB203580-sensitive pathway. The peptide coverages of the proteins and scores in the MASCOT analyses are shown in Table I. The ratios of signal intensities of these spots between three sample combinations by using images shown in Fig. 1 are also shown.

BAG2 and splicing factor arginine/serine-rich 9 (spots a and e) were significantly up-regulated in a p38-dependent manner just as Hsp27, a known substrate phosphorylated in the p38-MAPKAPK2 pathway, on spots c and d, suggesting that BAG2 and splicing factor arginine/serine-rich 9 are novel factors involved in the p38-dependent phosphorylation cascades. In this study, we focused on BAG2 as a candidate of p38-signaling factor and characterized its phosphorylation in the following experiments.

**BAG2 Phosphorylation Is Specifically Controlled by p38 MAPK**—To confirm that BAG2 was specifically phosphorylated by the p38 cascade in response to treatment of anisomycin, we used HeLa cells transiently overexpressed with N-terminal HA-tagged human BAG2. The transfected cells were treated with or without anisomycin in the presence or absence of specific protein kinase inhibitors. Consistent with previous reports, SB203580 inhibited the activation of MAPKAPK2 by p38 and thereby inhibited Hsp27 phosphorylation (23), SP600125 inhibited phosphorylation of c-Jun (24), and U0126 inhibited the activation of ERK1/2 (25)(Fig. 3A). These inhibitors did not affect the expression of HA-BAG2.

These total lysates were used for second dimension immunoblot with anti-HA antibody (Fig. 3B). We detected four spots (indicated as arrows a–d) of HA-BAG2 in control HeLa cells. Treatment with anisomycin resulted in a remarkable increase in the two spots (c and d) with more acidic pI values and a corresponding decrease in the remaining two spots (a and b). These shifts in pI values were significantly inhibited by preincubation of cells with SB203580 but neither with SP600125 nor with U0126. These results suggested that BAG2 was specifically phosphorylated by p38 MAPK cascades in response to anisomycin treatment.

**BAG2-Ser20 Is Directly Phosphorylated by MAPKAPK2 in Vitro**—To identify the phosphorylation site on BAG2 specifically phosphorylated in response to anisomycin, we scanned the whole peptide sequences of BAG2 of three distinct species (Homo sapiens, Mus musculus, and Danio rerio) for possible phosphorylation sites. Then we found a discriminative sequence in human BAG2 (residues 13–22) that completely matched with the consensus MAPKAPK2 phosphorylation site, Hyd-X-Arg-X-Ser, where Hyd is a bulky hydrophobic residue (Phe > Leu > Val > Ala) and Ser is a phosphorylation site (26). As illustrated in Fig. 4 with a schematic domain structure of human BAG2, this sequence motif in human BAG2 is perfectly conserved among three species.

We therefore constructed a vector plasmid expressing a mutant human BAG2 in which Ser20 was mutated to alanine to determine whether BAG2-Ser20 was directly phosphorylated by MAPKAPK2. Recombinant GST-BAG2, GST-BAG2-S20A, or GST-Hsp27 was incubated with recombinant MAPKAPK2 or ERK in the presence of [γ-32P]ATP in vitro. Fig. 5A shows that GST-BAG2-wt as well as GST-Hsp27 were strongly phospho-
rylated by MAPKAPK2 and that substitution of alanine for Ser20 completely abolished MAPKAPK2 phosphorylation of BAG2. In contrast, ERK did not phosphorylate GST-BAG2-wt at all. The kinase activity of ERK was confirmed strong phosphorylation of myelin basic protein (27) (data not shown). The result demonstrates that MAPKAPK2 directly phosphorylates BAG2 at Ser20.

**BAG2-Ser20 Is Phosphorylated in Vivo in a p38-dependent Manner** —To confirm that the phosphorylation of BAG2-Ser20 was the phosphorylation site in response to anisomycin in vivo, two-dimensional immunoblot was performed using HeLa cells expressing BAG2-wt or BAG2-S20A mutant. As shown in Fig. 5B, mutation at Ser20 dramatically inhibited the pI shifts (left panel) that were observed in wild type BAG2 in response to anisomycin treatment (right). Moreover, only two forms of BAG2 with more basic pI values (spots a and b) were detectable in HeLa cells expressing mutant BAG2, although four forms of BAG2 with different pIs were detectable in HeLa cells expressing wild type BAG2 (spots a–d). Therefore, it is highly likely that spots c and d correspond to BAG2 phosphorylated at Ser20 by p38-dependent pathways. These results demonstrated that BAG2 was phosphorylated at Ser20 in response to anisomycin treatment in vivo. Judging from the pattern of two-dimensional immunoblot, another phosphorylation site independent of p38 activation was suggested.

**MAPKAPK2 Phosphorylates BAG2 in Vivo in Response to Anisomycin Treatment**—MAPKAPK2 is activated downstream of p38 MAPK, and we have proved that recombinant MAPKAPK2 is able to phosphorylate GST-BAG2 in vitro (Fig. 5A). Next we generated siRNA targeting endogenous MAPKAPK2 to evaluate the role of MAPKAPK2 in the p38 MAPK cascade in respect to phosphorylation of BAG2. To ascertain the efficiency of siRNA, 293 cells cultured in 6-well plates were transfected with 250 or 500 ng of siMAPKAPK2. Two days after transfection, cell lysates were prepared and subjected to immunoblot with anti-MAPKAPK2 antibody (Fig. 6A). The result showed that 250 ng of siMAPKAPK2 were sufficient to suppress the endogenous expression of MAPKAPK2. SB203580 treatment of cells co-transfected with 500 ng of pEF-BOS/HA-BAG2 and 500 ng of control RNA (top panel) or 500 ng of siMAPKAPK2 (second panel). siMAPKAPK2 significantly reduced the intensities of spots c and d, suggesting the essential role of MAPKAPK2 in the anisomycin-dependent phosphorylation of BAG2. SB203580 treatment of cells co-transfected with 500 ng of pEF-BOS/HA-BAG2 and 500 ng of control RNA also diminished the spot intensities of a and b as already shown in Figs. 3 and 5. MAPKAPK2 level was also examined on the same blot showing the efficacy of siRNA-mediated inhibition of MAPKAPK2 expression. Nonspecifically reacted spots on the upper left corners showed the equal loadings of protein samples. The results indicate that significant portion of anisomycin-induced phosphorylation of BAG2 is mediated by MAPKAPK2.
Cell growth, differentiation, and apoptosis are regulated by diverse extracellular signals. The MAPK cascades integrate and process various extracellular signals by phosphorylating substrates, which alters their catalytic activities and conformations or creates binding sites for protein-protein interactions. To identify proteins that are involved in MAPK cascades, diverse approaches were developed. We have shown here that components of the p38 MAPK cascade can be identified by a proteomic approach. Our approach consisted of phosphoprotein purification, 2D-DIGE, and identification of proteins by peptide mass fingerprinting. This method enabled us to identify many spots unique to p38 MAPK-activated cells, which may be the candidates for the factors involved in the signaling pathway. Our approach is suitable to identify components that are phosphorylated within minutes after stimulation. Previous approaches such as transient expression of the active form of kinases in the cascade may not be appropriate to detect such factors. Interaction between a kinase and its substrates is generally not strong enough to pull down its substrates by immunoaffinity purification. A yeast two-hybrid system often produces false positives.

The enrichment of phosphoproteins may be effective to identify minor components such as factors involved in signal transduction, since when total cell lysates from p38 MAPK-activated and -suppressed cells were analyzed by 2D-DIGE under the same conditions, most of the red spots detected by using phosphoprotein-enriched samples were hardly visible hidden behind other spots of nonphosphorylated proteins (data not shown). We detected numerous red or orange spots at a higher molecular weight range (Fig. 1A). By using narrower pH ranges for the first dimension and polyacrylamide gels at different concentrations for the second dimension, more candidates will be detected. We are focusing our effort to identify these proteins by peptide mass fingerprinting.

We identified here BAG2 and splicing factor arginine/serine-rich 9 as potential substrates for the p38 MAPK cascade. BAG2 phosphorylation is specifically controlled by p38 MAPK pathways. HeLa cells transfected with pEFBOS-HA-BAG2 were treated with anisomycin in the presence or absence of kinase inhibitors, SB203580, SP600125, and U0126, and the lysates were subjected to two-dimensional immunoblot with anti-HA. A, SB203580 (indicated as SB) inhibition of phosphorylations of p38 MAPK substrates, MAPKAPK2 and Hsp27, as revealed by immunoblots using phosphospecific antibodies. Similarly, SP600125 and U0126 inhibited c-Jun and ERK phosphorylation, respectively. Anti-HA immunoblot shows the equal expression of HA-BAG2. B, two-dimensional immunoblots of HA-BAG2 of HeLa cell lysates as in A showing the shifts in pI values upon anisomycin treatment and specific inhibition of these shifts by SB203580.

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Fig. 3. BAG2 phosphorylation is specifically controlled by p38 MAPK pathways. HeLa cells transfected with pEFBOS-HA-BAG2 were treated with anisomycin in the presence or absence of kinase inhibitors, SB203580, SP600125, and U0126, and the lysates were subjected to two-dimensional immunoblot with anti-HA. A, SB203580 (indicated as SB) inhibition of phosphorylations of p38 MAPK substrates, MAPKAPK2 and Hsp27, as revealed by immunoblots using phosphospecific antibodies. Similarly, SP600125 and U0126 inhibited c-Jun and ERK phosphorylation, respectively. Anti-HA immunoblot shows the equal expression of HA-BAG2. B, two-dimensional immunoblots of HA-BAG2 of HeLa cell lysates as in A showing the shifts in pI values upon anisomycin treatment and specific inhibition of these shifts by SB203580.

Fig. 4. Predicted phosphorylation site by MAPKAPK2 and a schematic domain structure of human BAG2. Amino acid sequences of human, mouse, and zebrafish BAG2 surrounding a possible phosphorylation site by MAPKAPK2 (Hyd-X-Arg-X-Ser, where Hyd is a bulky hydrophobic amino acid (Phe > Leu > Val > Ala), Ser is the phosphorylation site, and X is any amino acid) are shown together with a schematic domain structure of human BAG2. The BAG domain (residues 109–189) and coiled-coil region (residues 20–82) are shown.

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splicing. If the activity of arginine/serine-rich 9 can be changed through phosphorylation, it would provide another example of regulation of mRNA metabolism by p38/MAPKAPK2 cascade.

BAG2 was phosphorylated in a p38 MAPK-dependent manner in response to anisomycin stimulation. An in vitro kinase assay clearly demonstrated that MAPKAPK2 phosphorylated BAG2 on Ser20. Moreover, by utilizing a Ser to Ala mutant, we confirmed that BAG2-Ser20 was phosphorylated in a p38 MAPK-dependent manner. The results of two-dimensional immunoblot using the BAG2-S20A mutant suggested that there was another phosphorylation site on BAG2. The detail of the phosphorylation is currently unknown. siRNA-mediated inhibition of MAPKAPK2 expression significantly but not completely decreased the shifts in pI of BAG2 (Fig. 6), suggesting that p38-dependent kinase(s) other than MAPKAPK2 exists. Possible candidates are other MAPKAPK family members, since their substrate specificity resembles that of MAPKAPK2 (28). Previous studies demonstrated that BAG family proteins are known as pivotal binding partner proteins of Hsc/Hsp70 molecular chaperones. All of the BAG1, BAG2, the BAG3 proteins interact with ATPase domain of Hsc/Hsp70 through the BAG domains, and suppress the chaperon activities of Hsc/Hsp70 in vitro (29). Furthermore, most of BAG family proteins play important roles in the regulation of apoptosis, cell survival, and stress response. For example, BAG4 (SODD) was identified as a binding partner for tumor necrosis factor receptor-1 (TNFR1), death receptor 3, and Bcl-2 and was shown to act in an antipapoptotic manner (30, 31). BAG3 was also reported as a regulator of stress-induced apoptosis in normal and neoplastic leukocytes (32). These previous results suggested the involvement of BAG2 in the regulation of apoptosis. We therefore extensively examined the effect of BAG2 expression using wild type and mutants of S20A and S20D (phosphomimetic). However, none of them accelerated or inhibited the anisomycin-induced apoptosis of HeLa cells so far examined (data not shown).

We confirmed the previous results and found that BAG2 constitutively bound to Hsp70 irrespective of the cellular stresses in vivo and that this tight binding to Hsp70 resulted in the strong inhibition of chaperon activity of Hsp70 in the refolding of denatured luciferase (data not shown). We also tested the effect of phosphorylation on the binding of BAG2 to Hsp70 using wild type and mutants of S20A and S20D (phosphomimetic). However, none of them accelerated or inhibited the anisomycin-induced apoptosis of HeLa cells so far examined (data not shown).

We confirmed the previous results and found that BAG2

FIG. 5. BAG2-Ser20 is phosphorylated by MAPKAPK2 in vitro, and its phosphorylation is essential for anisomycin-induced pI shifts. A, in vitro kinase assay performed using GST-BAG2 (BAG2-wt), GST-BAG2 with mutation at Ser20 to alanine (BAG2-S20A), or GST-Hsp27 (Hsp27) as a substrate and MAPKAPK2 or ERK2 as a kinase. The autoradiogram is shown. B, two-dimensional immunoblot with anti-HA antibody using HeLa cells expressing HA-BAG2-S20A (left panels) or HA-BAG2-wt (right panels). The shifts of pI values of BAG2 toward acidic pH (arrows c and d) in response to anisomycin treatment is absolutely undetectable in cells expressing HA-BAG2-S20A. CBB, Coomassie Brilliant Blue.

FIG. 6. MAPKAPK2 phosphorylates BAG2 in vivo in response to anisomycin treatment. siRNA targeting MAPKAPK2 (siMAPKAPK2) was transfected into HeLa cells to determine whether MAPKAPK2 is critical for BAG2 phosphorylation in vivo. A, transfection of both 250 and 500 ng of siMAPKAPK2 are sufficient to suppress endogenous expression of MAPKAPK2 (upper panel). Immunoblot (IB) with anti-actin antibody is shown for equal loading (lower panel). B, two-dimensional immunoblots with anti-MAPKAPK2 and anti-HA antibodies. Transfection of siMAPKAPK2 suppressed both endogenous expression of MAPKAPK2 and pI shifts of HA-BAG2 in response to anisomycin stimulation. To demonstrate the complete suppression of MAPKAPK2 expression, the blots were exposed for a rather longer period of time than in Figs. 3 and 5.
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which is the phosphorylation site by MAPKAPK-2. Since the coiled-coil domain functions in protein-protein interaction, it may be an interesting possibility that BAG2 interaction with other unidentified protein through the coiled-coil domain is regulated through the phosphorylation of BAG2 within the complex.

In this study, we have demonstrated the method constituting of phosphoprotein purification, 2D-DIGE, and mass spectrometric determination of the proteins for the identification of substrates for p38 MAPK cascades. Our approach could be applicable to any protein kinase of which a specific inhibitor substrates for p38 MAPK cascades. Our approach could be applicable to any protein kinase of which a specific inhibitor would be available. Therefore, this method will provide a wide range of applications to diverse biological phenomena.

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