Communication

LSP1 Is the Major Substrate for Mitogen-activated Protein Kinase-activated Protein Kinase 2 in Human Neutrophils*

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In intact cells, mitogen-activated protein kinase-activated protein (MAPKAP) kinase 2 is rapidly activated by various cytokines, stresses, and chemotactic factors. The small heat shock protein p27 has been shown to be a substrate for MAPKAP kinase 2. Recently, we identified a novel substrate, designated p60, for MAPKAP kinase 2 in human neutrophils (Zu, Y.-L., Ai, Y., Gilchrist, A., Labadia, M. E., Sha’afi, R. I., and Huang, C.-K. (1996) Blood 87, 5287–5296). To further understand the signaling pathway of MAPKAP kinase 2, we have purified p60 from a heat-treated neutrophil lysate by DEAE-cellulose chromatography and SDS-polyacrylamide gel electrophoresis. Microsequencing of five peptides derived from purified p60 indicates that p60 is lymphocyte-specific protein 1 (LSP1). Furthermore, antibodies specific for human and mouse LSP1 react with human and mouse p60. The sequence of human LSP1 indicates two serine residues at positions 204 and 252 as potential phosphorylation sites. The amino acid sequences surrounding these two sites are in agreement with the consensus sequence (Xaa-Xaa-Hyd-Xaa-Xaa-Ser-Xaa-Xaa) for phosphorylation by MAPKAP kinase 2. Both serine residues in human LSP1 and the corresponding conserved serine residues in mouse LSP1 are in the basic C-terminal F-actin binding domain. Various fusion proteins of wild type and truncated mouse LSP1 with glutathione S-transferase were tested for their capacity to be phosphorylated by MAPKAP kinase 2. The results indicate that LSP1 is a substrate for MAPKAP kinase 2 in vitro and that the phosphorylation sites are located in the basic C-terminal domain of LSP1. Because both the small heat shock proteins and LSP1 are F-actin binding proteins, these results suggest a role for MAPKAP kinase 2 in the regulation of cytoskeletal structure or function.

A variety of extracellular stimuli activate mitogen-activated protein kinases (MAPKs) through an intracellular kinase cascade, which includes MAP kinase kinase kinase and MAP kinase kinase. The MAPK family includes members of the p42/p44 MAPKs (ERK1 and 2), the p54 MAPKs (SAPKs or JNKs), and the p38 MAPKs (1–7).

MAP kinase-activated protein (MAPKAP) kinase 2 was originally identified as a substrate for the p42/p44 MAPKs in vitro (8). However, recent data indicate that in intact cells, the upstream kinase that regulates MAPKAP kinase 2 is p38 MAPK (9–12). Treatment of cells with endotoxin, interleukin-1, tumor necrosis factor, or various stress stimuli activate p38 MAPK and MAPKAP kinase 2 (9–12). In human neutrophils MAPKAP kinase 2 is also activated by the chemotactic factor fMet-Leu-Phe and phorbol 12-myristate 13-acetate (13). The function of MAPKAP kinase 2 is not known, but its upstream kinase p38 MAPK has been proposed to be involved in the biosynthesis of inflammatory cytokines (14), apoptosis (15), and platelet aggregation (16). MAPKAP kinase 2 contains a C-terminal autoinhibitory domain (17–19) and is activated by phosphorylation at multiple sites (18, 19).

MAPKAP kinase 2 can phosphorylate several proteins in vitro: rabbit skeletal muscle glycogen synthase (8) and tyrosine hydroxylase (20), whereas the low molecular weight heat shock proteins (HSP27 in human and HSP25 in mice) are substrates for MAPKAP kinase 2 in intact cells (21). Recently we observed that the major substrate for MAPKAP kinase 2 in human neutrophils is not HSP27 but is a protein termed p60 (13). In order to further understand the signaling pathway of MAPKAP kinase 2, we have purified p60 and shown that it is LSP1, a 339-amino acid cytoskeletal protein, the expression of which is restricted to neutrophils, lymphocytes, and macrophages (22–26).

EXPERIMENTAL PROCEDURES

Neutrophils were isolated from whole human blood using Ficoll/Hypaque gradients as described (13). A sample of $1 \times 10^6$ cells was suspended in lysis buffer containing 10 mM HEPES (pH 7.3), 11.5% sucrose, 1 mM EDTA, 1 mM EGTA, and 2 mM diisopropylfluorophosphate. After sonication (2 min), the cell lysate was centrifuged for 30 min at 13,500 $\times g$. The supernatant was then mixed in boiling water for 30 min and centrifuged for 10 min at 13,500 $\times g$. The supernatant was loaded on a DEAE-cellulose column (0.5 ml, pre-equilibrated in buffer A (30 mM HEPES (pH 7.3), 1 mM EDTA, and 1 mM EGTA)). The loaded column was washed with 4 ml of buffer A, and proteins were eluted sequentially from the column with 0.5-ml aliquots of buffer A containing 0.1 M, 0.2 M, 0.3 M, or 0.5 M NaCl. Aliquots from each fraction were assayed for p60 by phosphorylation by the constitutive MAPKAP kinase 2 mutant T334A as described (13).

RESULTS AND DISCUSSION

We have recently identified a novel substrate for MAPKAP kinase 2, designated p60, in intact neutrophils using the autoactive truncated mutant of MAPKAP kinase 2, T334A (13). Here we used this mutant to purify and characterize p60. Proteins in a human neutrophil lysate were phosphorylated by addition of T334A and $[\gamma^{32}\text{P}]$ATP as described (13). Fig. 1 shows that the addition of T334A results in increased phosphorylation of p60 after incubation for 10 or 20 min (lanes 1–5).
Purification of p60. p60 was purified from a heat-treated human neutrophil lysate. Proteins in a lysate from human neutrophils before (lanes 2–5) or after (lanes 6–8) heat treatment were phosphorylated with [γ-32P]ATP by the autoactive human MAPKAP kinase 2 mutant T334A. Phosphorylation was performed as described previously (13). The contents of the reaction mixture and the incubation times are indicated at the top.

Lanes 6–8 show that p60 is present and can be phosphorylated in the supernatant of heat-treated human neutrophils. Similar phosphorylation of p60 was observed in heat-treated extracts of mouse spleen and thymus but not in lysates of mouse kidney and heart (not shown). We took advantage of the heat stable nature of p60 and purified p60 from a heat-treated neutrophil lysate by DEAE-cellulose chromatography. Proteins were eluted from the DEAE-cellulose column with different concentrations of NaCl and assayed for the presence of p60 by phosphorylation with T334A. p60 eluted in the fractions containing 0.2 or 0.3 M NaCl (Fig. 2, lanes 4–6 and 11–13). Using 10⁶ neutrophils, approximately 140 μg of protein was present in the 0.2 M NaCl eluate. This sample was concentrated, and proteins were separated on a SDS-10% polyacrylamide gel. After a brief staining of the gel, gel pieces containing p60 (31 μg) were prepared and submitted to microsequencing (Biotechnology Laboratory, Yale University). The amino acid sequence of five peptides derived from p60 indicated that p60 is LSP1 (Fig. 3a). Inspection of the human LSP1 protein sequence (22) identifies two serine residues at positions 204 and 252 as potential phosphorylation sites. The amino acid sequences surrounding both sites are in agreement with the minimal sequence required for efficient phosphorylation by MAPKAP kinase 2 (27). Both serine residues are contained within the basic C-terminal domain, which is highly conserved between human and mouse LSP1 protein (24). The corresponding serine residues in mouse LSP1 are at positions 195 and 243, respectively (Ref. 25 and Fig. 3b). Inspection of the human LSP1 protein sequence (22) reveals a C-terminal 152 amino acid residues, as predicted from the full-length protein sequence (23). The sequences of five peptides obtained from purified p60 are underlined. Uncertain amino acid residues are shown in lowercase letters. b, the sequences of two putative MAPKAP kinase 2 phosphorylation sites in human and mouse LSP1 are aligned with the consensus phosphorylation site of MAPKAP kinase 2. The positions of the serine residues are indicated as a subscript.

**Fig. 3.** Sequencing results of the purified p60. a, the complete amino acid sequence of human LSP1 (23) is shown. The sequences of five peptides obtained from purified p60 are underlined. Uncertain amino acid residues are shown in lowercase letters. b, the sequences of two putative MAPKAP kinase 2 phosphorylation sites in human and mouse LSP1 are aligned with the consensus phosphorylation site of MAPKAP kinase 2. The positions of the serine residues are indicated as a subscript.
Antiserum was used at a final dilution of 1:2000. Each lane contains 10 ng of purified p60. Rabbit antiserum raised against mouse LSP1 (lanes 1 and 2) or human LSP1 (lanes 3 and 4) was used in the Enhanced Chemiluminescence system. Antiserum was used at a final dilution of 1:2000. Each lane contains 10 ng of purified p60.

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