Induction of Synaptosomal-Associated Protein-23 kD (SNAP-23) by Various Cytokines

Cytokines manifest their function through regulation of gene expression. We searched for immediate-early cytokine responsive genes by the mRNA differential display technique using interleukin-3 (IL-3)-dependent OTT-1 cells, and have isolated a novel cDNA which encodes 210 amino acids and shows 87% amino acid identity to human SNAP-23 (synaptosomal-associated protein of 23 kD). The message for this protein (mouse SNAP-23) was induced in OTT-1 cells by IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-5. The experiment using C-terminal deletion mutants of the common \( \beta \) subunit (\( \beta \)c) of IL-3/GM-CSF/IL-5 receptors showed that expression of SNAP-23 was associated with the Ras-Raf-MAPK pathway, but not with the JAK-STAT pathway. Moreover, SNAP-23 was induced in response to a wide variety of cytokines, including IL-2, IL-3, IL-5, IL-10, stem cell factor, G-CSF, GM-CSF, leukemia inhibitory factor, and erythropoietin. Constitutive expression of SNAP-23 was seen in various tissues, including heart, lung, kidney, liver, spleen, and small intestine. Possible involvement of SNAP-23 in cytokine signal transduction is discussed.

1998 by The American Society of Hematology.

MATERIALS AND METHODS

Cells. IL-3-dependent murine myelomonocytic cell line OTT-1, pro-B cell line Ba/F3, mast cell line MC9, and myeloid progenitor cell lines L-G3 and L-GM1 were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 2 ng/mL murine IL-3. The murine erythroid cell line SKT6 and myeloid leukemia cell line M1 were cultured in RPMI 1640 medium containing 10% FCS. The IL-2-dependent T-cell line CTLL-2 was cultured in RPMI 1640 medium containing 10% FCS and 1 mmol/L IL-2.

From the Department of Anatomy and Neurobiology, Wakayama Medical School, Wakayama; the Department of Hemopoietic Factors, the Institute of Medical Science, University of Tokyo; and the Laboratory of Cellular Biosynthesis, the Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan.

Supported by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science and Culture, Japan. The Department of Hemopoietic Factors is partly supported by Chugai Pharmaceutical Company Ltd.

Address reprint requests to Yoshihiro Morikawa, MD, Department of Anatomy and Neurobiology, Wakayama Medical School, Kiyobancho-27, Wakayama 640, Japan.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1998 by The American Society of Hematology.

0006-4971/98/9201-0033$3.00/0

From www.bloodjournal.org by guest on November 11, 2017. For personal use only.
a Dye Terminator Cycle Sequencing Ready Reaction kit from Perkin-Elmer Cetus Corp.

Isolation of the full-length cDNA. A partial cDNA fragment of mouse SNAP-23 obtained by differential display was labeled with digoxigenin (DIG) by using a DIG-DNA labeling kit (Boehringer Mannheim, Indianapolis, IN) and used for colony hybridization screening of the cDNA library from OTT-1 cells stimulated with IL-3 according to the manufacturer’s instructions. The longest clone (A2-2) was sequenced by using synthetic oligonucleotide primers.

**RESULTS**

Isolation of genes induced by IL-3/GM-CSF/IL-5. To obtain genes whose induction is mediated by IL-3, GM-CSF, and IL-5, we used a cytokine-dependent cell line, OTT-1, which responds to IL-3, GM-CSF, and IL-5, and the mRNA differential display technique. We obtained mRNA from OTT-1 cells before and 1 hour after stimulation with IL-3, GM-CSF, and IL-5, and performed mRNA differential display using them. cDNA bands detected in OTT-1 cells stimulated with IL-3, GM-CSF, and IL-5 were excised from the display gel and cloned into the pCRII TA vector. To eliminate false-positive clones, the induction of the mRNA expression was examined in the OTT-1 cells stimulated with IL-3 and GM-CSF by Northern blot analysis using the EcoRI fragment of these clones as a probe. When we used the clone 2.1 as a probe, 2.2 kb of mRNA was induced after stimulation with IL-3 and GM-CSF, and IL-5 was excised from the display gel and cloned into the pcRII TA vector. To eliminate false-positive clones, the induction of the mRNA expression was examined in the OTT-1 cells stimulated with IL-3 and GM-CSF by Northern blot analysis using the EcoRI fragment of these clones as a probe. When we used the clone 2.1 as a probe, 2.2 kb of mRNA was induced after stimulation with IL-3 and GM-CSF, but not in them without stimulation (Fig 1), indicating that clone 2.1 mRNA was induced after stimulation with IL-3 and GM-CSF.

Structure of protein encoded by the 2.1 cDNA. The initial 2.1 cDNA clone obtained from the differential display contained about 250 bp of coding region of the mRNA. To obtain a full-length cDNA, we screened 1 × 10^6 independent clones from a cDNA library of IL-3–stimulated OTT-1 cells with randomly DIG-labeled cDNA for clone 2.1 and obtained 12 positive clones. Four clones among them were sequenced (Fig 2). Three clones, A2-2, A6-1, and A6-2, contained an ATG codon followed by a single open reading frame, and had an in-frame stop codon upstream of the ATG, but clones A6-1 and A6-2 had some deletion at the end of 3′-untranslated region.

The sequence of clone A2-1 matched to a fragment between nucleotides 350 to 1890 of the longest clone A2-2. The cDNA sequence of A2-2 encodes a predicted protein of 210 amino acids, and the cystein-rich region between residues 79 and 87, to which palmitate is linked by thioester bonds, was found (Fig 2A). A search for the nucleotide data base, DDBJ, showed that clone A2-2 is a mouse homologue of SNAP-23 and shows 98.6% similarity and 86.7% identity to human SNAP-23 at the amino acid level (Fig 2B).

Induction of the SNAP-23 message by stimulation with IL-3, GM-CSF, and IL-5. SNAP-23 induction by stimulation with IL-3, GM-CSF, and IL-5 was examined by Northern blotting using OTT-1 and L-GM3 cells (Fig 3). In OTT-1 cells, induction of SNAP-23 by IL-3 began 1 hour after stimulation, reached a maximal level at 2 hours, and was detected at 12 hours. SNAP-23 was also induced after stimulation with GM-CSF and IL-5 in the same manner. In L-GM3 cells, which proliferate in response to IL-3 and differentiate in response to GM-CSF, a high level of SNAP-23 induction was observed 1 hour after stimulation with both IL-3 and GM-CSF.
Induction of the SNAP-23 message mediated by the membrane-distal region of the common β (βc) subunit of IL-3/GM-CSF/IL-5 receptor. We also examined induction of SNAP-23 in other IL-3–dependent cell line Ba/F3. In Ba/F3 cells, SNAP-23 mRNA was induced by IL-3 at earlier time points than in OTT-1 cells; its induction began 20 minutes after stimulation, reached the maximal level at 1 to 2 hours, and thereafter decreased to the basal level in 12 hours (Fig 4A).

The common β (βc) subunit of the IL-3, GM-CSF, and IL-5 receptors is responsible for signal transduction of these cytokines; a membrane-proximal region of the cytoplasmic domain is responsible for activation of the JAK-STAT pathway and the distal region is required for activation of the Ras-Raf-MAP kinase cascade. To address whether the induction of SNAP-23 is mediated by the membrane-proximal or -distal region, deletion mutants of the common β subunit of hGM-CSFR and established the Ba/F3 stable transfectants expressing the wild-type hGM-CSFR and a series of mutant β subunits. 7,15,16 The induction of SNAP-23 was detected in BaF3/bc589, BaF3/bc626, and BaF3/bc (wild type) 2 hours after stimulation with hGM-CSF, but not in BaF/bc415, BaF/bc517, and BaF/bc445 (Fig 4B). This result shows that the membrane-distal region (544-589), which is required for...
activation of the Ras-Raf-MAP kinase cascade, is also required for the induction of SNAP-23.

Induction of SNAP-23 message by stimulation with other cytokines inducing proliferation and/or differentiation. As described above, SNAP-23 was induced by stimulation with IL-3, GM-CSF, and IL-5. We also examined the induction of SNAP-23 by stimulation with SCF and IL-10 in an IL-3-dependent mast cell line MC9, G-CSF in myeloid progenitor cell line L-G3, leukemia inhibitory factor (LIF) in a LIF-responsive myeloid leukemia cell line M1, EPO in erythroid cell line SKT6, and IL-2 in IL-2-dependent T-cell line CTLL-2 (Fig 5). When MC9 was stimulated with IL-3, SCF, and IL-10, which lead to cell proliferation, SNAP-23 was induced in the same manner; induction began 20 minutes after the stimulation and high level of the expression was kept until 2 hours after the stimulation. Furthermore, we also detected the induction of SNAP-23 in CTLL-2 cells stimulated with IL-2.

When SKT6 cells, which proliferate without EPO and undergo limited erythroid differentiation in response to EPO, were stimulated with EPO, SNAP-23 was induced 2 hours after stimulation. In addition, SNAP-23 was induced by stimulation with LIF in M1 cells.

In L-G3 cells, which differentiate into neutrophils in response to G-CSF, G-CSF as well as IL-3 induced SNAP-23 1 hour after the stimulation. Thus, SNAP-23 was induced by a wide range of cytokines regardless of biological consequences induced by cytokines.

Expression of SNAP-23 message in tissues. SNAP-23 expression was examined in various mouse tissues. While SNAP-23 expression requires the stimulation of cytokines in hematopoietic cell lines, a high level of SNAP-23 mRNA was detected without stimulation in various tissues including heart, lung, liver, skeletal muscle, kidney, skin, stomach, and small intestine (Fig 6). The SNAP-23 message was also detected in spleen, smooth muscle, and pancreas at lower intensity. In the brain and the testis, expression of SNAP-23 message was marginal. Such a wide distribution of SNAP-23 suggests important roles of SNAP-23 in various tissues as well.

DISCUSSION

We previously identified two novel genes, CIS and OSM, induced by various cytokines through the JAK-STAT pathway by using a cDNA library subtraction technique.9–11 In this study we searched for the genes differentially or commonly induced by IL-3, GM-CSF, and IL-5 by using the mRNA differential display technique and obtained several novel genes. Here we describe a gene, SNAP-23, which is commonly induced by IL-3, GM-CSF, and IL-5. SNAP-23 is also induced by several other cytokines, including IL-2, IL-10, EPO, SCF, G-CSF, and LIF. Despite the inducible expression of SNAP-23 by various cytokines in vitro, SNAP-23 is widely expressed in various tissues without any stimulation. The constitutive expression of SNAP-23 message in vivo may be due to various cytokines existing in the body.

SNAP-23 is an isoform of SNAP-25 which is one of the SNARE proteins.19 Docking and fusion of the cytoplasmic vesicles to the cell membrane involves the formation of a trimeric core complex of three proteins, vesicle-associated membrane protein (VAMP), syntaxin, and SNAP-25,20 in transport of proteins along the secretory pathway of eukaryotic cells. Although different isoforms of VAMP and syntaxin have been reported in many cell types,20–24 SNAP-25 has been exclusively detected in neuronal tissues.25 On the other hand, human SNAP-23 is ubiquitously expressed.19 A mouse homolog of SNAP-23 we cloned in this study is widely expressed in most tissues. Thus, it is likely that SNAP-23 is functionally equivalent to SNAP-25 and replaces SNAP-25 in nonneuronal tissues.

Cytokine receptors transmit their signals through multiple signaling pathways. Among them, Ras activation pathway is well characterized.26–28 Ras is activated after formation of Shc-Grb-SOS complex or tyrosine phosphorylation of Vav. Activation of Ras then leads to induction of c-fos and c-jun via
activation of Raf and MAPK. Activation of the Ras-Raf-MAPK cascade is induced by most cytokines in all hematopoietin receptors with some exception, such as IL-4. Another well-characterized signaling pathway is the JAK-STAT pathway, which is activated by virtually all cytokines. In the IL-3/GM-CSF/IL-5 receptor systems, the binding of the ligand to the receptor results in rapid tyrosine phosphorylation of Jak2, which leads to the induction of some genes, including OSM and CIS, via activation of STAT5. These two pathways require the distinct region of the βc subunit of IL-3/GM-CSF/IL-5 receptors; membrane-proximal and -distal regions of βc subunit activate JAK-STAT pathway and Ras-Raf-MAPK cascade, respectively. Deletion analysis using truncated βc subunit of the IL-3/GM-CSF/IL-5 receptors suggested that SNAP-23 is one of the target genes downstream of the Ras-Raf-MAP kinase cascade. Alternatively, induction of SNAP-23 is mediated by a yet uncharacterized pathway that interacts with the distal region of the βc subunit which is also required for activation of the Ras-Raf-MAPK pathway.

Shimazaki et al reported protein kinase C (PKC)-mediated phosphorylation of SNAP-25. In addition, recent reports suggest that PI3 kinase, whose activation also requires the membrane-distal region of the βc subunit, activates the PKC. Alternatively, induction of the SNAP-23 by cytokines, SNAP-23 may also be phosphorylated by PKC downstream of cytokine receptors to become functionally active.

In summary, we have shown that mouse SNAP-23 is induced by stimulation with various cytokines, implicating important roles of SNAP-23 in cytokine-mediated function. However, it remains to be investigated whether SNAP-23 simply functions as a protein for the fusion of vesicle and plasma membrane, or if it also participates in signal transduction. Some investigators reported that the SNARE proteins participate in the cytokinesis and the resealing of the plasma membrane as well. Therefore, one may speculate that SNAP-23...
also plays a role in cellular functions other than exocytosis such as cell proliferation or differentiation. In fact, SNAP-23 message was induced in the process of both proliferation and differentiation. The generation of the antibody against SNAP-23 and mice lacking the SNAP-23 gene will provide a clear insight into the role of SNAP-23 induced by cytokines in hematopoietic cells. After we completed this work, Araki et al.\textsuperscript{34} reported that the mouse SNAP-23, which is the same as the one we cloned, interacts with syntaxin 4 for the translocation of GLUT4 vesicles to the plasma membrane in adipocytes.

**Fig 5.** Induction of SNAP-23 by the stimulation with various cytokines. Factor-dependent cell lines MC9, CTLL-2, and L-G3 were deprived of serum and growth factors, then stimulated with mIL-3, mSCF, mIL-10, mIL-2, or hG-CSF for the indicated time (h). SKT6 cells and M1 cells were stimulated with hEPO and mLIF for the indicated time (h), respectively. Poly(A)$^+$ RNA (1 μg/lane) was separated from the cells and blotted with SNAP-23 and G3PDH probes.

**Fig 6.** Distribution of SNAP-23 mRNA in various mouse tissues. Poly(A)$^+$ RNA (2 μg/lane) from various mouse tissues was hybridized with the SNAP-23 probe.
ACKNOWLEDGMENT

The nucleotide sequence data reported in this paper will appear in the DNA Data Bank of Japan, European Molecular Biology Laboratory, and GenBank nucleotide databases with the following accession number: AB007444.

REFERENCES

Induction of Synaptosomal-Associated Protein-23 kD (SNAP-23) by Various Cytokines

Yoshihiro Morikawa, Hitoshi Nishida, Kazuhide Misawa, Tetsuya Nosaka, Atsushi Miyajima, Emiko Senba and Toshio Kitamura