Human Neutrophil Lactoferrin trans-Activates the Matrix Metalloproteinase 1 Gene through Stress-activated MAPK Signaling Modules*

Received for publication, August 13, 2001, and in revised form, August 28, 2001
Published, JBC Papers in Press, September 4, 2001, DOI 10.1074/jbc.M107724200

Sang-Muk Oh, Dae Hyun Hahn‡, Ik-Hwan Kim, and Sang-Yun Choi§

From the Division of Life Sciences, Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea

It has been proposed that human neutrophil lactoferrin (Lf) could be involved in gene expression as a DNA-binding protein after its translocation into the nucleus. However, the molecular basis of Lf action has not been defined, and Lf-regulated target genes have not been identified. We report here that overexpressed Lf functions as a specific trans-activator of matrix metalloproteinase 1 (MMP1) gene, and that induction of this AP-1-responsive gene is mediated via the stress-activated MAPK signaling modules. Transactivation of the MMP1 promoter by overexpressed Lf requires the presence of an AP-1 binding site. In gel shift experiments, Lf did not interact directly with AP-1-containing fragments of the MMP1 promoter. However, nuclear extracts from Lf-expressing cells contained increased levels of proteins that bound to AP-1 elements. This Lf-induced AP-1 DNA binding activity was reduced by a p38 MAPK inhibitor. Inhibitors of the MEK kinases had little effect on Lf-induced AP-1. However, expression of dominant-negative MKK4 or JNK1 inhibited Lf-induced gene expression. The JNK activity stimulated by Lf correlates with the enhanced AP-1 binding ability. These findings demonstrate that the Lf-induced activation of AP-1 is mediated via JNK and p38 MAPK pathways.

Human Lf derived from neutrophils is secreted in high concentration in colostrum but is normally present in low concentration in most other exocrine fluids, such as saliva, tears, bile, and pancreatic fluid (1). In addition to the iron binding capacity of Lf, a variety of other biological functions are attributed to Lf by as yet unknown mechanisms. For example, Lf exerts several effects on the inflammatory and immune responses against various tumors (2). During this process, circulating levels of Lf increase significantly. On the other hand, Lf has been reported to act as an inhibitor of cytokine response in vitro at a very low concentration by suppressing the release of cytokines such as interleukins and tumor necrosis factor (3). Lf also may act as a potent activator of natural killer cells and a direct inhibitor of tumor cell growth (4). Therefore, the balanced synthesis and exocrine secretion of the Lf in the cell suggest its precise roles in various biological systems.

A previous report revealed that Lf enters the cell from the serum and is transported into the nucleus where it binds to DNA (5). Recently random DNA sequences capable of binding to Lf have been demonstrated, which may imply an Lf-mediated regulation of gene expression (6). However, little information on naturally occurring target genes that are directly responsive to Lf is available. Therefore, we have investigated one of the roles of Lf as a multifunctional regulatory protein. To do so, the exogenous human neutrophil Lf gene was overexpressed in various mammalian cells. There is considerable interest in the role of Lf because it acts as a trans-activator for the expression of a subset of responsive genes, particularly AP-1-inducible genes such as fibroblast collagenase-1, matrix metalloproteinase 1 (MMP1), and monocyte chemoattractant protein 1 (MCP-1). The MMP1 exerts important effects on physiological and pathological conditions of cells including tumor metastasis (7), although its exact mechanism remains unclear.

In this study, we have focused on the elucidation of the molecular mechanism by which the overexpressed Lf mediates the stimulation of MMP1 production. Nested deletion and point mutations have allowed us to narrow down the Lf-responsive promoter region of the MMP1 gene to a specific element. By performing EMSA, we have attempted to see whether Lf could interact directly or indirectly with the promoter of the target gene. To test potential roles for specific signaling pathways in mediating Lf-induced gene expression, we have used several specific kinase inhibitors and dominant-negative mutant forms of kinase cDNAs. We demonstrate here that the activation of stress-activated MAPK signaling pathways by Lf plays a crucial role in the up-regulation of the MMP1 gene.

EXPERIMENTAL PROCEDURES

Coll Culture and Transfection Assays—Balb/c-3T3, HeLa, Jurkat, and COS-1 cells were purchased from American Type Culture Collection (Manassas, VA). The cells were grown and transfected using DEAE-dextran (8), an equal amount (5 μg) of the human neutrophil Lf expression vector pLf, and CAT construct. Cotransfection with the pRSV-β-galactosidase plasmid (0.5 μg per transfection) and cytofluorometric analysis of β-galactosidase activity were performed to normalize for transfection efficiency. CAT activities were quantitated with a liquid scintillation counter. CAT assay data are averages of at least three separate experiments. Long term Lf-expressing cell lines, B-hLf2 and H-hLf6, were cloned by stably cotransfecting Balb/c and HeLa cells with either pLf or pCI-neo, respectively, by the calcium phosphate method.
geneticin (G418). Both B-hLf2 and H-hLf6 cells were maintained in the complete medium supplemented with 0.5 mg/ml G418. Geneticin, TPA, poly(dI-dC), acetyl-CoA, SB203580, PD98059, and U0126 were pur-

Plasmids—Plasmid pLf was the generous gift of P. Furmanski (6). Plasmid pColICAT-517 and MMP1 probe were generously provided by P. Angel (10). The 5′ deletion mutant plasmids pColICAT-517, pCol-

Preparation of Nuclear Extracts and EMSAs—Nuclear extracts of Balb/c-pCI-neo and B-hLf2 cells were prepared by the method of Andrews and Faller (15). EMSAs were performed as described previously (12). Oligonucleotides with an AP-1 consensus binding site (5′-CGCT-

RESULTS

To identify Lf-responsive promoters, a set of putative gene promoters linked to the reporter gene CAT was cotransfected with an Lf expression vector (pLf). Transcriptional activities of the MMP1 and MCP-1 gene promoters (Fig. 1A, compare pCol-

Zymography Assay—After washing cells with cold phosphate-buff-

Reverse Transcription-PCR (RT-PCR)—Total RNA was isolated from HeLa or H-hLf6 cells by the guanidinium thiocyanate-cesium chloride extraction method (12). Reverse transcription and PCR amplification were per-

β-galactosidase activity in intact cells and functions in trans.

The promoter of the TPA-inducible MMP1 gene was further analyzed to identify a cis-acting element for the Lf. A putative responsive region on the MMP1 gene promoter was identified by deletion analysis. Nested deletion of the MMP1 gene promoter linked to the reporter gene CAT up to the TPA-responsive element (TRE) (−73 to −67, TGAAGTC) preserved a moderately high inducibility of the chimeric gene by Lf (Fig. 2A). The pColICAT-73 construct spanning the −73/+63 region, which contains an active TRE, showed the weakest Lf-respon-
trans-Activation of Matrix Metalloproteinase 1 Gene by Lf

Purified Lf was incubated with each labeled oligonucleotide containing an Lf binding consensus (Lf oligo), AP-1 consensus (AP-1 oligo), and MMP1 promoter fragments containing the −73/+63 region (MMP1). EMSA was carried out as described under "Experimental Procedures." B, enhanced AP-1 binding activity in cells expressing Lf. Nuclear extracts were prepared from either Balb/c-pCl-neo (−) or B-hLf2 cells (+). Balb/c-pCl-neo cells treated with 20 nM TPA for 4 h (TPA) were used as a positive control. For the specific competition, a 50-fold molar excess of unlabelled AP-1 consensus competitor (AP-1) or MMP1 gene promoter fragments containing a TRE (MMP1) was added in each indicated reaction.

Many transcription factors including c-Jun, c-Fos, and ATF2 are effectors of three distinct groups of MAPKs: extracellular signal-regulated kinase, p38 MAPK, and JNK. To determine whether Lf-induced AP-1 activation possibly resulted from activation of any one of the MAPK pathways, the effect of specific kinase inhibitors (SB203580, PD98059, and U0126) was tested. As measured by EMSAs using nuclear extracts from B-hLf2 cells treated with SB203580, which is a specific inhibitor of p38α (SAPK2α) and its isoform p38β (SAPK2b) but which has no inhibitory effect on SAPK3 and SAPK4 (17), AP-1 DNA binding activity was reduced by about 50% as compared with control extracts (from cells that received only Me2SO) (Fig. 4A). This result suggests that Lf-induced AP-1 activation is, at least in large part, p38 MAPK-mediated. In contrast, both PD98059 (18) and U0126 (19), which specifically inhibit phosphorylation of MKK1 and MKK2, had little effect on the AP-1 activity. MKK4 is an essential component of the JNK signal transduction pathway and also activates p38 MAPK (20). Therefore, it was anticipated that interfering with the MKK4 activity by overexpression of the dominant-negative MKK4 (SEK1) might cause the inhibition of downstream pathways implicated in both JNK and p38 MAPK signaling pathways. Fig. 4B demonstrates that the transient cotransfection of Balb/c cells with pLf and dominant-negative SEK1 selectively blocked AP-1 binding, indicating the involvement of stress-activated MAPK signaling modules in Lf-induced AP-1 activation. Furthermore, the expression of dominant-negative JNK1 inhibited the ability of Lf to trans-activate the MMP1 (Fig. 4C). To confirm that the activated AP-1 observed in cells expressing Lf resulted from the activated JNK, the phosphorylation levels of c-Jun in lysates were determined. In B-hLf2 cells, JNK strongly phosphorylated Ser-73 of the c-Jun NH2-terminal domain (Fig. 4D, upper panel). Moreover, Lf expression in B-hLf2 cells caused a marked increase in JNK1 protein kinase activity (Fig. 4D, lower panel).
were used for each assay. B-hLf2 cells were incubated with each MAPK inhibitor, SB203580 (10 μM), PD98059 (50 μM), or U0126 (10 μM). Nuclear extracts for EMSA were prepared from Balb/c-pCI-neo (-) or B-hLf2 cells (+) treated with each inhibitor or only its vehicle Me2SO (DMSO). Before the treatment, cells were starved for 24 h in Dulbecco’s modified Eagle’s medium containing 0.5% fetal bovine serum. A, AP-1 binding activity and effects of dominant-negative SEK1. An EMSA was performed as described above. Either dominant-negative SEK1 (SEK1-DN) or pCDNA3 backbone plasmid was used for the transient co-transfection of Balb/c cells with an equal amount of pLf (+) or pUC19 (−). C, effects of dominant-negative JNK1 (JNK1-DN) on trans-activation. Relative CAT activity was determined after co-transfection of HeLa cells with the indicated plasmids. Shown is the mean ± S.E. for three independent experiments in duplicates. D, JNK activity in cells expressing Lf. Immunoblot analysis (upper panel) using phospho-c-Jun (p-c-Jun) (Ser-73) antibody and in vitro kinase assay (lower panel) for JNK1 were performed as described under “Experimental Procedures.” Total cell lysates obtained from Balb/c-pCI-neo (-) or B-hLf2 (+) cells were used for each assay.

**DISCUSSION**

Human Lf has been implicated in various biological processes including the regulation of cell growth and inflammation as well as the primary defense against bacterial infection (21). Lf has been considered to exert a control function in the balance of cellular components as a nuclear factor (6, 22). It has been demonstrated that sequence-specific binding of Lf to DNA occurs under stringent conditions. Here we demonstrate that overexpressed Lf is capable of regulating the expression of the MCP-1 and MMP1 genes, which are implicated in inflammation and metastasis (23, 24). Moreover, we found that Lf specifically trans-activates MMP1, an AP-1-inducible gene, by altering the DNA binding properties of AP-1 in both fibroblasts and T lymphocytes. The biological significance of the up-regulation of the MMP1 gene by Lf is unclear. Circulating levels of Lf increase significantly during the inflammatory process (2). Additionally activated leukocytes and many tumor cells secrete large amounts of the metalloproteinases (25). Therefore, it is likely that the activation of MCP-1 and MMP1 genes in response to Lf may be involved in physiological and pathological processes such as inflammation and tumorigenesis (3, 26).

In this study, we also found that Lf required a TRE site for the trans-activation of the MMP1 gene and that AP-1 binding activity was substantially enhanced in cells expressing Lf. AP-1 activity in cells is known to be regulated by upstream signal transduction pathways such as the MAPK cascades (20). Therefore, it seems likely that Lf-mediated AP-1 activation might be mediated via known signaling networks. Our studies with specific kinase inhibitor drugs and dominant-negative components of the MKK4 pathway indicated that both JNK and p38 MAPK might contribute to Lf-induced AP-1 activation. Consistent with a role for MKK4 in Lf-responsive AP-1 activation, the MKK4 effector kinase JNK was activated by Lf. Together these data strongly suggest that SAPK and c-Jun may mainly mediate the Lf-induced signaling pathway. We do not rule out the possibility that the SAPK can also activate other transcription factors such as Elk1, MEF2c, and ATF2 so that their subsequent transcriptional and/or posttranslational targets in turn may contribute indirectly to the activation of AP-1 in cells expressing Lf (27).

The transcriptional targets of the Lf-mediated SAPK signaling pathway have not been fully established. However, we have observed that human neutrophil Lf greatly up-regulated AP-1-inducible inflammatory mediator cytokines and some particular genes in the immunoglobulin gene superfamily (2). Although the biological functions of Lf have been controversial (3, 22, 28), our new results suggest an important role for Lf in gene regulation and eventually cellular activity. We demonstrated that the constitutively overexpressed Lf induced the MKK4-SAPK signaling pathway leading to AP-1 activation and MCP-1 expression. The increased expression of chemotactants such as MCP-1 by the AP-1 causes leukocyte recruitment, resulting in the enhancement of the inflammatory response by releasing the protease MMP1 (25). This AP-1-induced expression of MMP1 is likely to have a number of consequences, including remodeling of matrix, oxidative burst, and cell injury (29). It is likely that the circulating level of Lf is an important factor for the regulation of Lf-induced genes. Indeed, the level of Lf on natural killer cell cytotoxicity depends on the level of Lf (30).

Furthermore, multihormone signaling pathways may be involved in modulating Lf gene activity and its circulating level, and the activated human blood lymphocytes may express Lf receptors (31). The autocrine property of the Lf molecule and its role in the specific gene activation imply the involvement of Lf receptors in further upstream signaling immediately following binding of Lf to its receptor. Therefore, studies are under way to investigate whether the expression level of Lf and its translocation through the receptor will be crucially related with the cellular activity in the response to a variety of stresses.

---

2 S.-M. Oh and S.-Y. Choi, manuscript in preparation.
Acknowledgments—We thank C. Vaziri for helpful suggestions and critical comments on the manuscript and R. Conrad for critical reading of the manuscript.

REFERENCES
1. Davidson, L., and Lonnerdal, B. (1989) Am. J. Physiol. 257, G930–G934
2. Baynes, R., Bezwoda, W., Khan, Q., and Mansoor, N. (1986) Scand. J. Haematol. 36, 79–84
3. Machnicki, M., Zimecki, M., and Zagulski, T. (1993) Int. J. Exp. Pathol. 74, 433–439
4. Bezault, J., Bhimani, R., Wiprovnick, J., and Furmanski, P. (1994) Cancer Res. 54, 2310–2312
5. Garre, C., Bianchi-Scarra, G., Sirito, M., Musso, M., and Ravazzolo, R. (1992) J. Cell. Physiol. 153, 477–482
6. He, J., and Furmanski, P. (1995) Nature 373, 721–724
7. Birks, H. (1995) Curr. Opin. Cell Biol. 7, 728–735
8. Lopata, M. A., Cleveland, D. W., and Sollner-Webb, B. (1984) Nucleic Acids Res. 12, 5707–5717
9. Wilson, L., Flyer, D., and Faller, D. (1987) Mol. Cell. Biol. 7, 2406–2415
10. Angel, P., Baumann, I., Stein, B., Delius, H., Rahmsdorf, H., and Herrlich, P. (1992) Mol. Cell. Biol. 12, 5707–5717
11. Damiens, E., Mazurier, E., El Yazidi, I., Mazurier, E., Duthille, I., Spik, G., and Boilly-Marer, Y. (1990) J. Cell. Biochem. 32, 486–498
12. Graves, D. T., Jiang, Y. L., Williamsen, M. J., and Vallele, A. J. (1989) Science 245, 1490–1493
13. Watanabe, H., Nakane, H., Yamashita, K., Hayakawa, T., and Okada, Y. (1993) J. Cell Sci. 104, 991–999
14. Herlaar, E., and Brown, Z. (1999) Mol. Med. Today 5, 439–447
15. Gupta, S., Campbell, D., Derijard, B., and Davis, R. J. (1995) Science 267, 389–392
16. Andrews, N., and Faller, D. (1991) Nucleic Acids Res. 19, 2499
17. Rangaswamy, J., Gupta, S., Dickens, M., and Han, J. (1995) J. Biol. Chem. 270, 7420–7426
18. Cuenda, A., Reuse, J., Doza, Y., Meier, R., Cohen, P., Gallagher, T., Young, P., and Lee, J. (1995) FEBS Lett. 364, 229–233
19. Alessi, D., Cuenda, A., Cohen, P., Dudley, D., and Saltiel, A. (1995) J. Biol. Chem. 270, 27489–27494
20. Davis, R. J. (2000) Cell 103, 239–252
21. Baveye, E., Elaiss, E., Mazurier, J., and Legrand, D. (2000) FEBS Lett. 469, 5–6
22. Damiens, E., El Yazidi, I., Mazurier, E., Duthille, I., Spik, G., and Boilly-Marer, Y. (1999) J. Cell. Biochem. 74, 486–498
23. Graves, D. T., Jiang, Y. L., Williamsen, M. J., and Vallele, A. J. (1989) Science 245, 1490–1493
24. Watanabe, H., Nakane, H., Yamashita, K., Hayakawa, T., and Okada, Y. (1993) J. Cell Sci. 104, 991–999
25. Herlaar, E., and Brown, Z. (1999) Mol. Med. Today 5, 439–447
26. Liotta, L., Steeg, P., and Stetler-Stevenson, W. (1991) Cell 64, 327–336
27. Galcheva-Gargova, Z., Derijard, B., Wu, I. H., and Davis, R. J. (1994) Science 265, 806–808
28. Ping, P., and Murphy, E. (2000) Circ. Res. 86, 921–922
29. Babier, B. M. (2000) Am. J. Med. 109, 33–44
30. Damiens, E., Mazurier, E., El Yazidi, I., Masson, M., Duthille, I., Spik, G., and Boilly-Marer, Y. (1998) Biochim. Biophys. Acta 1402, 277–287
31. Faucheux, B., Nillesse, N., Damier, P., Spik, G., Mouatt-Pirgent, A., Pierce, A., Leveugle, B., Kubis, N., Hauw, J., Agid, Y., and Hirsch, E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9658–9667
Human Neutrophil Lactoferrin trans-Activates the Matrix Metalloproteinase 1 Gene through Stress-activated MAPK Signaling Modules
Sang-Muk Oh, Dae Hyun Hahm, Ik-Hwan Kim and Sang-Yun Choi

J. Biol. Chem. 2001, 276:42575-42579.
doi: 10.1074/jbc.M107724200 originally published online September 4, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107724200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 13 of which can be accessed free at http://www.jbc.org/content/276/45/42575.full.html#ref-list-1