Vol. 199, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1994 Pages 26-32

MOLECULAR CLONING AND CHARACTERIZATION OF G-CSF INDUCED GENE cDNA

Miyuki Shimane, Kenzaburo Tani*, Kazuo Maruyama#, Satoshi Takahashi, Keiya Ozawa and Shigetaka Asano

Hematology and Oncology, Institute of Medical Science, University of Tokyo, Tokyo 108, Japan

Department of Hygiene and Oncology, Tokyo Medical and Dental University, School of Medicine, Tokyo 113, Japan

Received January 4, 1994

SUMMARY G-CSF (granulocyte colony-stimulating factor) is known to specifically stimulate the production and the functional activation of neutrophils. To investigate the intracellular signaling pathway of myeloid cells stimulated by G-CSF, we isolated new genes whose expression was induced by G-CSF. First of all, we constructed λgt10 cDNA library from G-CSF-stimulated mononuclear cells (MNC) of a chronic myelogeneous leukemia (CML) patient (CML-MNC) and screened the cDNA library by a differential hybridization method. The 24 candidate clones which specifically hybridized with G-CSF-stimulated CML-MNC cDNA probes, but not with unstimulated CML-MNC cDNA probes, were obtained after $8x10^4$ individual clones had been screened. One of these clones, GIG-1 (G-CSF-induced gene-1), was further characterized. The size of the GIG-1 mRNA was about 0.9kb. The GIG-1 mRNA was expressed mainly in the myeloid leukemic cell lines.

G-CSF(granulocyte colony- stimulating factor) is a member of hematopoietic growth factors family and plays important roles in the regulation of the proliferation, differentiation, and functional activation of granulocytes (1,2). Recently, recombinant human G-CSF has been widely used in the clinical field as a very potent drug to increase the number of granulocytes (2). In addition, the cDNA and chromosomal gene of the human and murine G-CSF receptors have been cloned (3-5), and the functional domains of the G-CSF receptor have been reported (6). Furthermore, it has also been revealed that G-CSF regulates the expression of the myeloid marker genes, myeloperoxidase and alkaline phosphatase which accompany morphological differentiation from myeloblasts to

* To whom correspondence should be addressed.

Abbreviations: G-CSF, granulocyte colony-stimulating factor; CML, chronic myelogeneous leukemia; MNC, mononuclear cells; GIG-1,G-CSF-induced gene-1.

0006-291X/94 $5.00

granulocytes (7). The intracellular molecular mechanisms which follow the binding of G-CSF to the G-CSF receptor and result in proliferation and differentiation of myeloid cells, however, still remain unknown.

We have described the isolation and nucleotide sequence of a new gene whose expression is induced by G-CSF in myeloid cells which we investigated for the purpose of studying the intracellular signaling pathways which follow G-CSF stimulation. As there are no established human myeloid cell lines which clearly show in vitro morphological differentiation by G-CSF stimulation, in this study we used the bone marrow mononuclear cells of a chronic myelogeneous leukemia (CML) patient in chronic phase, which are well known to be proliferated and differentiated in response to G-CSF in a manner similar to normal cells and are able to be obtained in large numbers with relative ease.

Materials and Methods

Cell source and poly(A)$^+$ RNA isolation After obtaining the informed consent, bone marrow cells were collected with heparinized syringe from the iliac crest of locally anesthetized CML patients in chronic phase. After Ficoll-Hypaque density gradient centrifugation, fractionated MNC were cultured in IMDM(Iscove's Modified Dulbbecco's Medium supplemented with 10% fetal calf serum (FCS) at 37℃, with or without the addition of 50 ng/ml of recombinant human G-CSF (Chugai Pharmaceutical Co.,Ltd., Tokyo, Japan). After the cells were cultured for 18 hours, total RNA was extracted by the AGPC(acid guanidine phenol chloroform) method (8), and subsequently the poly(A)$^+$RNA was purified using oligo(dT) latex (Takara Syuzou, Kyoto, Japan).

Construction of the cDNA library From 2.8μg of poly(A)$^+$ RNA of CML-MNC which were preliminary stimulated by 50 ng/ml G-CSF for 18 hours, a cDNA library was prepared as previously described(9). Briefly, a first-strand cDNA was synthesized with oligo(dT) primers, and the double-stranded cDNA was synthesized from the first strand cDNA mainly using the cDNA synthesis kit (Amersham, Buckinghamshire, UK). The synthesized cDNA was then ligated to *a* λgt10 vector with a λgt10 cloning system (Bethesda Research Laboratories., Gaithersburg, Md.). The library was packaged in vitro using packaging extract(Stratagene, La Jolla, CA).

Screening of the cDNA library and cloning of full length cDNA The 1st and the 2nd screening were performed following the differential hybridization method as described elsewhere(9). Briefly, CML-MNC G(+) λgt10 cDNA library was first plated with E.Coli C600hfl. Two nylon filters (Amersham, Buckinghamshire, UK) for each phage plate were lifted as described before (9). Following the standard method, a cDNA hybridization probe was prepared from 1μg of poly(A)$^+$ RNA of CML-MNC cultured with or without 50 ng/ml of G-CSF for 18 hours. Plaques that gave a signal with a G(+) but not with G(-) probe were selected (G(+) clones). These clones were further screened by Northern blot analysis. Namely, only the clones which hybridized with CML-MNC G(+) mRNA, but not CML-MNC G(-) mRNA were selected for further studies of DNA sequencing.

In order to clone the full length cDNA, the original library was rescreened by plaque hybridization with probes which were insert cDNA of clone 5-31 and the synthesized oligomer from sense sequence of 5'end of cDNA of clone 6-21 (Fig. 3, Sense1, 25mer). Then, 5'end of GIG-1 cDNA was obtained from 1μg of poly(A)$^+$ RNA of CML-MNC G(+) by new PCR cloning method developed for the cloning of 5' end of cDNA which has the advantage of the cap structure of mRNA(10). The cap structure of 5'end of mRNA was removed with tobacco acid pyrophosphatase and was replaced by synthesized oligomer (Fig.3, P1, 25mer,

GAGAGAGACAGGCCTTCTTGGCCGA) which bound to the cloning vector of pME18SCG. The 1st strand cDNA of this mRNA was synthesized with reverse transcriptase primed at the antisense primer of GIG-1 cDNA (Fig.3, P2), and was amplified with primers P1 and P3 (Fig.3, 43mer, GAGAGAGAGGCCTGGGTG GCCGTGCTCAAAGCAATCAGGCAGA) which included the antisense sequence of GIG-1 cDNA. This PCR product was digested with Sfi I and was cloned into pME18SCG.

DNA sequencing and computer analysis The cDNA inserts of positive clones were subcloned into pBluescript SK^- and SK^+ vector (Stratagene,La Jolla, CA) and were sequenced using the dideoxy chain termination method. DNA sequencing data were analyzed using EMBL and GenBank data bases. Hydropathy plot analysis was done following the method of Kyte Doolittle (11).

Northern blot analysis 10 or 20μg of total cellular RNA from cultured or non-cultured cells were electrophoresed in 1% agarose gel containing formaldehyde and then transferred onto nitrocellulose filters (MSI, Westborough, Mass). The filters were hybridized with [^{32}P]-labeled EcoRI cDNA fragments of positive clones in hybridization buffer containing 50% formamide at 50℃ for 18 hours. After hybridization, the filters were washed and autoradiographed as previously described (12).

Results and Discussion

Cloning and DNA sequencing G-CSF induced gene cDNAs were cloned by the differential hybridization method. After a 1st screening of $8x10^4$ independent clones of the λgt10 cDNA library derived from CML-MNC cultured for 18 hours with G-CSF, 40 individual candidate clones that hybridized only with the G(+) cDNA probes were obtained, and 24 of them were selected after the at 2nd screening. After the 3rd screening by Northern blot analysis, one cDNA clone (No.5-31) was selected because of its selective hybridization with mRNA obtained from G-CSF stimulated CML-MNC (Fig.1). The gene for clone 5-31 was named GIG-1 (G-CSF-Induced Gene-1). As the estimated size of GIG-1 mRNA was about 0.9kb by Northern blot analysis as shown in Fig.1, the insert length (0.6kb) of the clone 5-31 was not long enough for full length GIG-1 cDNA. The longer clone (6-21) of 0.7kb was obtained after further screening of 10^5 independent clones from the non-amplified original library by the clone 5-31, and thereafter clone 7-1 was obtained from another 10^5 independent clones by mean of a synthesized oligonucleotide probe which recognized the 5' end sense sequence of the clone 6-21 as shown in Fig.3.

The partial nucleotide sequence of GIG-1 cDNA was obtained after DNA sequencing of clones 5-31, 6-21 and 7-1. The enzyme restriction map for GIG-1 cDNA is shown in Fig.2. From the DNA sequencing results, clone 5-31 (498bp) lacked 95bp and at the 106th nucleotide and 73bp at the 5' end compared with clone 6-21 (676bp). This difference might be due to the results of alternative splicing of the GIG-1 gene. Clone 7-1 spanned longer than clone 6-21 by 95bp at the 5' end. The cDNA sequence of clone 7-1 contained only one long open reading frame, but was still not long enough for the estimated size of GIG-1 mRNA of 0.9kb. Therefore we tried to clone and determine 5' end of GIG-1

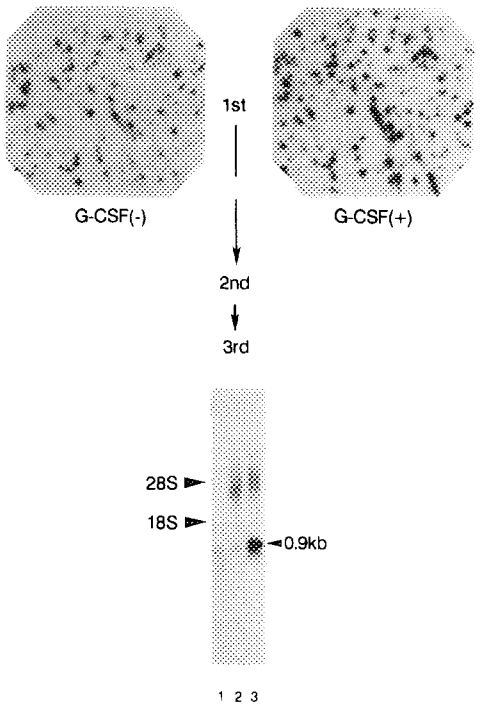


Fig.1. Process of GIG-1 cDNA cloning. The positive candidate clone picked up after 1st screening was shown with an arrow and was named as clone 5-31. The Northern blot analysis using a candidate (clone 5-31) cDNA probe showed a specific hybridized band only in G-CSF-stimulated CML-MNC (lane3), whereas no hybridized bands were observed either in unstimulated cultured CML-MNC (lane2) or uncultured CML-MNC (lane1).

cDNA by the use of the 5' capping method (10), these results revealed a further 64bp sequence at the 5' end of clone 7-1 (Fig.2). In addition to the theoretical background(interest) of this new method, the putative translation sequence of

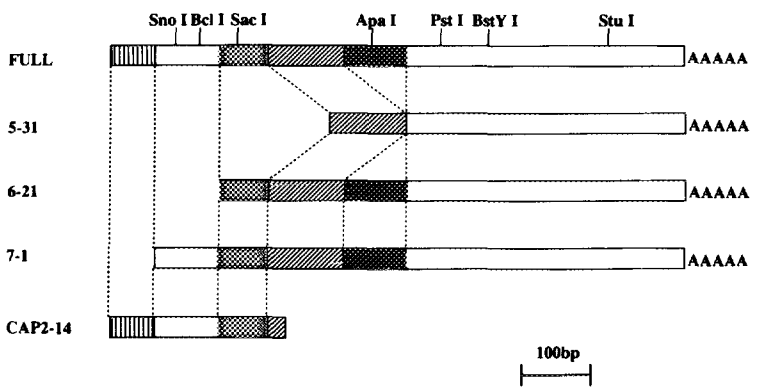


Fig.2. Restriction enzyme maps of GIG-1 cDNA clones. DNA sequencing information for the full length GIG-1 cDNA clone derived from clones 5-31, 6-21, 7-1 and CAP2-14. Each of the boxes with the same mark depicts the same DNA sequences.

CCCCATGG at position 177-184 almost matched Kozak's consensus sequence (13). These results strongly suggested that ATG codon at 181 was translation initiation site of GIG-1 cDNA, and the 833bp nucleotide sequence shown in Fig.3 was full length GIG-1 cDNA.

Predicted amino acid sequence of GIG-1 The amino acid sequence predicted from the 833bp nucleotide sequence is also shown in Fig.3. The open reading frame of GIG-1 cDNA encodes 165 amino acids and no glycosylation sites were suspected from this sequence. Fig.4 shows a hydropathy plot of GIG-1 protein. This shows GIG-1 protein has four repetitive hydrophobic protein regions. This pattern resembles the integral membrane protein of L6 which is considered to be related to cellular proliferation and tumorigenesis, and belongs to a family of integral membrane proteins (14). Therefore, the GIG-1 protein is hypothesized to be one of the membrane proteins at present. According to von Heijne (15), the cleavage site by signal peptidase will be located between the 22nd residue of alanine and the 23th residue of leucine in GIG-1 protein. To confirm

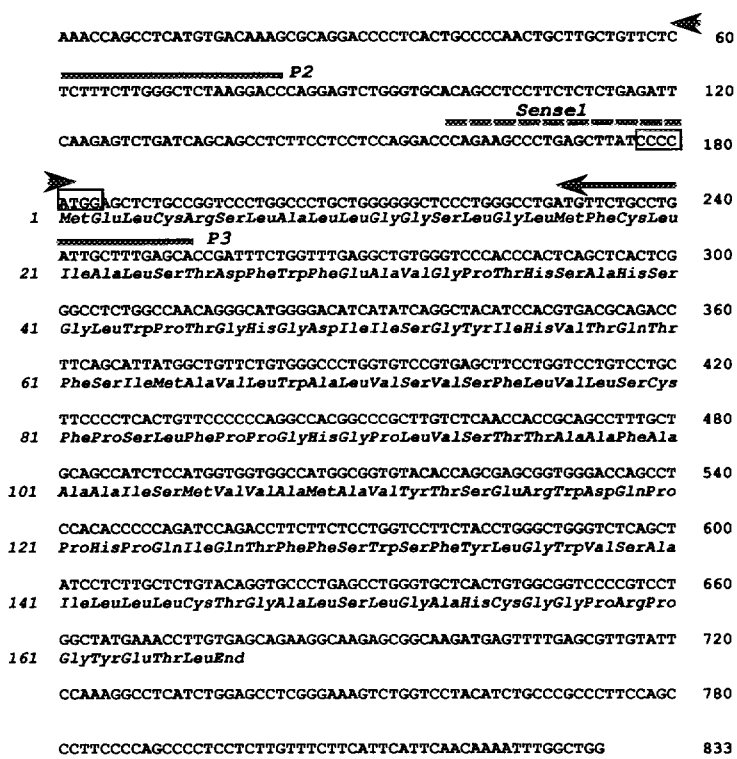
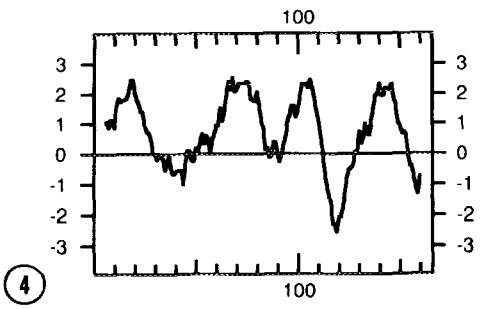


Fig.3. Nucleotide sequence with predicted amino acid sequence of GIG-1 full length cDNA. CCCCATGG which includes the translation initiation site of ATG is boxed. The antisense primer (P2) against the nucleotide sequence with arrow was used at the 1st strand cDNA synthesis before PCR, and the antisense primer for PCR included the sequence marked with P3 . The sense primer (Sense1) was used for the screening of the CML-MNC G(+) λgt10 cDNA library.



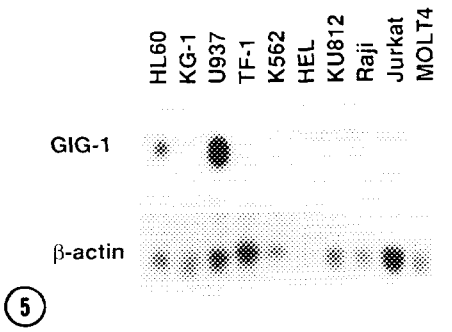


Fig.4. Hydropathy plot of the predicted GIG-1 amino acid sequence. The hydropathy plot was performed following the method of Kyte and Doolittle(13).

Fig.5. Northern blot analysis of GIG-1 mRNA expression in human hematopoietic cell lines. 20 μg each of the total RNAs were applied.

this hypothesis, the determination of the NH2-terminal amino acid sequence of the GIG-1 mature protein would be required.

Constitutive expression of GIG-1 Northern blot analysis of human hematopoietic cell lines was performed in order to survey constitutive expression of GIG-1 mRNA. As shown in Fig.5, GIG-1 mRNA was expressed in the myeloid cell lines HL-60, KG-1 and U937, but not in erythroid cell lines TF-1, K562, HEL and KU812, nor in the lymphoid cell lines Raji, Jurkat, and MOLT4. These results suggested that GIG-1 mRNA was mainly expressed in the myeloid cells.

During the preparation of this manuscript, computer analysis using the GenBank database showed the DNA sequence of NKG7 cDNA (16) was highly homologous to that of GIG-1 cDNA. NKG7 cDNA was cloned from the NK cell line B22, and its nucleotide sequence was different from that of GIG-1 by only four bases. Because of this dissimilarity, the predicted amino acid sequence was quite different between NKG7 and GIG-1 particularly at their carboxyl terminals, which deduced NKG7 gene to be for an integral membrane protein. This difference might simply be due to artificial results in the course of cDNA cloning and DNA sequencing, or might have some biological significance in distinguishing granulocytes from NK cells. The identification of the lineage of NK cells is controversial. It is interesting to note a recent report (17) which suggested that the $CD34^+$ $CD33^+$ Lin^- cell population could differentiate to myeloid cells as well as NK cells in the presence of certain growth factors. Considering the functional similarities and dis- similarities between granulocytes and NK cells, the existence of a common progenitor is possible. Further studies including the elucidation of the genomic structure of GIG-1 and functional analysis the GIG-1 protein should reveal a significant role for GIG-1 in granulocytes as well as NK cells.

Acknowledgments: We thank Drs. Sigekazu Nagata and Takashi Yokota for helpful discussion and also Drs. Mitsuo Nishikawa and Keisuke Takahashi for technical advice and support. This work was supported in part by research grants from the Ministry of Education, Science and Culture and the Ministry of Welfare of Japan.

References

1) Metcalf, D. (1989) Nature 339, 27-30.
2) Asano, S. (1991) Am. J. Pediatr. Hematol. Oncol. 13,400-413.
3) Fukunaga, R.,Ishizaka-Ikeda, E., Seto, Y.,and Nagata, S. (1990) Cell 61, 341.
4) Fukunaga, R., Seto, Y., Mizushima, S. and Nagata, S. (1990) Proc. Natl. Acad. Sci. USA 87, 8702.
5) Seto,Y., Fukunaga, R. and Nagata,S. (1990) J. Immunol. 146, 259-266.
6) Fukunaga, R., Ishizuka-Ikeda, E., Pan, C. X., Seto, Y.and Nagata, S. (1991) EMBO. J. 10, 2855-2865.
7) Lübbert,M.,Herrmann,F. and Koeffler,P. (1991) Blood 77, 909-924.
8) Moscinski,L.C.and Prystowsky, M.B.(1990) Oncogene 5, 31-37.
9) Maniats, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York.
10) Maruyama, K. and Sugano, S. (1993) Gene, in press.
11) Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105-132.
12) Morishita, K., Tsuchiya, M.,Asano, S., Kaziro, Y., Nagata, S. (1987) J. Biol. Chem. 262, 15208-15213.
13) Kozak,M. (1986) Cell 44, 283-292.
14) Marken, J. S., Schieven, G. L., Hellström, K. E., and Aruffo,A. (1992) Proc. Natl. Acad. Sci. USA 89, 3503-3507.
15) Von Heije, G. (1986) Nucl. Acids Res.14, 4683-4690.
16) Turman, M.A., Yabe, T., McSherry, C., Bach, F. H., Houchins, J.P. (1993) Hum. Immunol. 36, 34-40.
17) Shibuya, A., Nagayoshi, K., Nakamura, K., and Nakauchi, H. (1993) Blood 82, (supple. 1) 102a.