Generation of an Angiostatin-like Fragment from Plasminogen by Stromelysin-1 (MMP-3)†

H. R. Lijnen,*,‡ F. Ugwu,‡ A. Bini,§ and D. Collen‡

Center for Molecular and Vascular Biology, University of Leuven, Leuven, Belgium, and Laboratory of Blood Coagulation Biochemistry, The Lindsley F. Kimball Research Institute, The New York Blood Center, New York, New York 10021

Received December 30, 1997; Revised Manuscript Received February 10, 1998

ABSTRACT: Matrix metalloproteinase-3 (MMP-3 or stromelysin-1) specifically hydrolyzes the Glu^{59}−Asn^{60}, Pro^{447}−Val^{448}, and Pro^{544}−Ser^{545} peptide bonds in plasminogen, yielding a 55 kDa NH_2-terminal angiostatin-like domain (comprising kringles 1−4), a 14 kDa domain comprising kringle 5, and a 30 kDa domain comprising the serine proteinase domain. The conversion is completely abolished in the presence of the MMP inhibitors EDTA or 1,10-phenanthroline. Biospecific interaction analysis indicates that binding of proMMP-3 and MMP-3 to plasminogen occurs with comparable affinity (K_A of 4.7×10^6 and 4.1×10^6 M^{-1}, respectively) and is mediated via the miniplasminogen moiety (kringle 5 plus the proteinase domain) and via the catalytic domain of MMP-3. Thus, proteolytic cleavage of plasminogen by MMP-3 generates angiostatin-like fragments.

Plasminogen, the zymogen of the fibrinolytic enzyme plasmin, is a single chain glycoprotein of 92 kDa consisting of 791 amino acids (*1, 2*). Conversion to the two-chain plasmin molecule occurs by specific cleavage of the Arg^{561}−Val^{562} peptide bond by plasminogen activators (*3*) and may be associated with conversion of the native Glu-plasminogen molecule (NH_2-terminal glutamic acid) to modified forms (Lys-plasminogen) as a result of hydrolysis of the Arg^{68}−Met^{69}, Lys^{77}−Lys^{78}, or Lys^{78}−Val^{79} peptide bonds by plasmin and removal of the preactivation peptide (*4, 5*). Both chains of plasmin (approximately 65 and 25 kDa) are connected by two disulfide bonds, and the active site located in the 25 kDa COOH-terminal proteinase domain is composed of His^{603}, Asp^{646}, and Ser^{741} (*1*). The NH_2-terminal domain consists of five kringle structures, of which the lysine-binding sites located in the kringle 1−3 region play a key role in the regulation of fibrinolysis (*6, 7*). Recently, it has been reported that angiostatin, a plasminogen fragment containing kringles 1−4, has antiangiogenic properties (*8*). In vitro, angiostatin inhibits endothelial cell proliferation (*8*−*11*), and in mice in vivo it inhibits primary tumor growth and angiogenesis-dependent growth of metastases (*8, 11, 12*). The exact composition of the active angiostatin fragment as well as the mechanism by which it is generated in vivo is, however, still debated.

Matrix metalloproteinases (MMP's)[1] are also secreted as zymogens (proMMP's) that are activated in the extracellular milieu; active MMP's degrade most proteins and proteoglycans that constitute the extracellular matrix and thereby play a role in normal tissue remodeling and in pathological conditions such as arthritis, tumor invasion, and metastasis (*13*−*16*).

In the present study, we report that matrix metalloproteinase-3 (stromelysin-1) specifically generates angiostatin-like fragments from plasminogen.

MATERIALS AND METHODS

Proteins, Reagents, and Assays. Human plasminogen, plasmin, miniplasminogen (miniPlg), LBS I, LBS II, and the inactive plasminogen variant with substitution of the active site Ser^{741} by Ala, Plg(S741A), were obtained and characterized as described elsewhere (*17, 18*).

Recombinant human proMMP-3 (57 kDa native form) and MMP-3 (23.5 kDa truncated form, lacking the COOH-terminal domain) were a kind gift of Dr. H. Nagase (Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City) (*19, 20*). Protein concentrations were determined with the BCA protein assay (Pierce, Rockford, IL). The monoclonal antibodies MAb-42B12 (directed against plasminogen kringle 5), MAb-31E9 (directed against the proteinase domain), and MAb-34D3 (directed against LBS I) were obtained as described previously (*21, 22*).

The chromogenic substrate S-2403 for plasmin was purchased from Chromogenix (Antwerp, Belgium) and 1,10-phenanthroline from Sigma Chemie (Bornem, Belgium).

†This study was supported by grants from the Flemish Fund for Scientific Research (FWO, contract G.0126.96) and from the IUAP (contract P4/34).

* Address correspondence to this author at the University of Leuven, Campus Gasthuisberg O&N, Herestraat 49, B-3000 Leuven, Belgium. Tel: +32-16-34 57 71. Fax: +32-16-34 59 90. E-mail: roger.lijnen@med.kuleuven.ac.be.

‡ University of Leuven.

§ The New York Blood Center.

[1] Abbreviations: MMP-3, matrix metalloproteinase-3 (stromelysin-1; EC 3.4.24.17); MAb, monoclonal antibody; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Plg(S741A), recombinant plasminogen with the active site Ser^{741} substituted with Ala; miniPlg, plasminogen fragment consisting of kringle 5 and the proteinase domain; LBS I, plasminogen fragment consisting of kringles 1−3; LBS II, plasminogen fragment consisting of kringle 4; S-2403, D-Glp-Phe-Lys-*p*-nitroanilide.

S0006-2960(97)03179-6 CCC: $15.00
Published on Web 03/19/1998

The plasmin inhibitor Val-Phe-Lys-CH_2Cl was custom synthesized at UCB (Brussels, Belgium).

SDS−PAGE without reduction or after reduction with 1% dithioerythritol was performed on 10−15% gradient gels using the Phast system. Apparent molecular weights were determined from reduced gels, by comparison with a protein calibration mixture consisting of phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Densitometric scanning of SDS−PAGE was performed with the Gel-scan accessory of the Beckman DU60 spectrophotometer. Immunoblotting of nonreduced SDS−PAGE was performed according to Towbin (*23*).

NH_2-terminal amino acid sequence analysis was performed on an Applied Biosystems model 477A protein sequencer (Foster City, CA), interfaced with an Applied Biosystems model 120A on-line analyzer. Samples were electrophoresed on 10−20% Tris-HCl Ready Gels (Mini-Protean^R II Cell; Bio-Rad, Hercules, CA) and blotted on ProBlott membranes (Applied Biosystems).

Association rate constants (k_{assoc} in $M^{-1}\cdot s^{-1}$) and dissociation rate constants (k_{dissoc} in s^{-1}) for the interaction between (pro)MMP-3 and different plasminogen moieties were determined by real-time biospecific interaction analysis using the BIAcore instrument (Pharmacia, Biosensor AB). ProMMP-3 or MMP-3 were immobilized on the surface of sensor chip CM5 using the Amine Coupling kit (Pharmacia), and binding of plasminogen moieties was evaluated as described in detail elsewhere (*24*).

Proteolytic Cleavage of Plasminogen by MMP-3. Human plasminogen (final concentration 10 μM) was incubated with MMP-3 (final concentration 1 μM) at 37 °C in 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, 10 mM $CaCl_2$, and 0.01% Tween 80. At different time intervals (0−4 h), samples were removed from the incubation mixtures, EDTA (final concentration 25 mM) was added to stop the reaction, and SDS−PAGE was performed under nonreducing or reducing conditions. The amount of the different plasminogen fragments in the samples was determined by densitometric scanning of the gels, and the area of each fragment was expressed as percent of the total area at each time point.

RESULTS

Incubation of plasminogen with MMP-3 (enzyme/substrate ratio of 1/10) resulted in quantitative conversion to nondisulfide bonded fragments with molecular masses of 55, 40, 30, and 14 kDa, as shown by SDS−PAGE under nonreducing or reducing conditions. Addition of EDTA (final concentration 25 mM) or 1,10-phenanthroline (final concentration 1 mM) to the incubation mixture completely abolished proteolytic cleavage, confirming that it is MMP-dependent (not shown). Densitometric scanning of SDS−PAGE of samples taken at different time points revealed rapid generation of the 55 and 40 kDa fragments, with more than 50% of the plasminogen cleaved within 30 min. The 30 and 14 kDa fragments appeared somewhat later (Figure 1, inset A), while nearly complete conversion of plasminogen within 4 h was evident from the parallel decrease of the concentration of intact protein.

NH_2-terminal amino acid sequence analysis of samples after 1 h incubation with MMP-3 revealed a double sequence

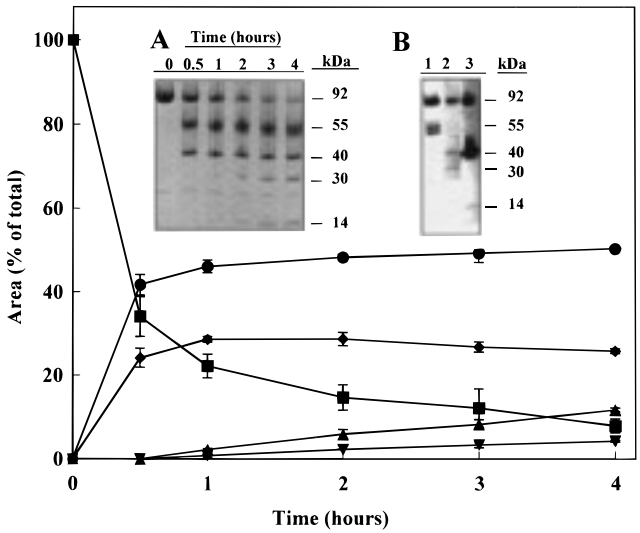

FIGURE 1: Time course of proteolytic cleavage of plasminogen by MMP-3 (enzyme/substrate ratio of 1/10). The area corresponding to intact plasminogen (■) or to the 55 kDa (●), 40 kDa (◆), 30 kDa (▲), and 14 kDa (▼) fragments at different time points was determined by densitometric scanning of reduced SDS−PAGE (inset A) and expressed as percent of the total area. The data represent mean ± SEM of three separate experiments. Inset B shows immunoblotting of nonreduced SDS−PAGE with MAb-34D3 (lane 1), MAb-31E9 (lane 2), and MAb-42B12 (lane 3).

for the 55 kDa fragment of plasminogen, starting at Glu^1 (about two-thirds of the protein) or Asn^{60} (one-third of the protein), respectively (Table 1). The 40, 30, and 14 kDa fragments showed one homogeneous sequence starting at Val^{448}, Ser^{545}, and Val^{448}, respectively. These data are compatible with cleavage of the Pro^{447}−Val^{448} peptide bond, generating the 55 kDa fragment comprising kringles 1−4; additional cleavage of the Glu^{59}−Asn^{60} peptide bond removed most of the preactivation peptide from about one-third of the protein. In addition, cleavage of the Pro^{447}−Val^{448} peptide bond generates the 40 kDa fragment (kringle 5 plus the proteinase domain), part of which is further degraded to the 30 kDa (proteinase domain) and 14 kDa (kringle 5) fragments by cleavage of the Pro^{544}−Ser^{545} peptide bond. The composition of the different molecular mass fragments is further confirmed by immunoblotting using monoclonal antibodies which are specific for kringle 5 (MAb-42B12), for LBS I (MAb-34D3), or for miniplasminogen ((MAb-31E9) (Figure 1, inset B). Indeed, MAb-42B12 reacts with residual intact plasminogen and with the 40 and 14 kDa fragments, confirming that they contain kringle 5. MAb-31E9 reacts with plasminogen, and with the 40 and 30 kDa fragments, confirming the presence of the proteinase domain. MAb-34D3 reacts with plasminogen and with the 55 kDa fragment, confirming that they contain at least kringles 1−3. The apparent heterogeneity of the 55 kDa fragment is in agreement with the double amino acid sequence (Table 1) and may also reflect carbohydrate heterogeneity in the plasminogen preparation (*17*).

Binding of different plasminogen fragments to proMMP-3 and to MMP-3 was evaluated by biospecific interaction analysis (Table 2). The affinity of Plg(S741A), active-site blocked plasmin, and miniplasminogen for binding to proMMP-3 and MMP-3 was comparable [K_A of (4.2−9.8) $\times$ 10^6 M^{-1}], whereas LBS I and LBS II did not bind. This suggests that the (pro)MMP-3 binding site is comprised

Table 1: NH_2-Terminal Amino Acid Sequence Analysis of Plasminogen Treated with MMP-3 (Enzyme/Substrate Ratio 1/10) for 1 h at 37 °C

	amino acid (recovery in pmol) in cycle							
fragment	1	2	3	4	5	6	7	8
55 kDa	Glu (30)	Pro (33)	Leu (22)	Asp (20)	Asp (28)	Tyr (26)	Val (22)	Asn (22)
	Asn (18)	Arg (11)	Lys (16)	Ser (7)	Ser (9)	Ile (9)	Ile (11)	Ile (17)
40 kDa	Val (40)	Val (41)	Leu (37)	Leu (37)	Pro (31)	Asp (23)	Val (27)	Glu (24)
30 kDa	Ser (4)	Phe (9)	Asp (8)	Cys (−)	Gly (8)	Lys (5)	Pro (7)	Gln (4)
14 kDa	Val (19)	Val (20)	Leu (15)	Leu (16)	Pro (14)	Asp (5)		

Table 2: Apparent Affinity Constants of ProMMP-3 (57 kDa) and MMP-3 (23.5 kDa) for Binding to Different Molecular Forms of Plasminogen[a]

	pro-MMP-3			MMP-3		
ligand	k_{assoc} ($\times 10^3$) ($M^{-1}\cdot s^{-1}$)	k_{dissoc} ($\times 10^{-3}$) (s^{-1})	K_A ($\times 10^6$) (M^{-1})	k_{assoc} ($\times 10^3$) ($M^{-1}\cdot s^{-1}$)	k_{dissoc} ($\times 10^{-3}$) (s^{-1})	K_A ($\times 10^6$) (M^{-1})
Plg(S741A)	83 ± 14	18 ± 2.3	4.7 ± 0.23	120 ± 42	29 ± 3.8	4.1 ± 1.3
VFK-plasmin[b]	64 ± 6.6	16 ± 0.8	4.2 ± 0.53	82 ± 5.9	17 ± 3.3	5.0 ± 0.67
miniPlg (VFK)[b]	72 ± 8.3	15 ± 0.7	4.7 ± 0.35	93 ± 32	9.2 ± 1.1	9.8 ± 1.9
LBS I	<1			<1		
LBS II	<1			<1		

[a] Data are mean ± SEM of three experiments. [b] Experiments performed in the presence of Val-Phe-Lys-CH_2Cl.

within the miniplasminogen moiety. Furthermore, comparable binding of the plasminogen moieties to 57 kDa proMMP-3 and 23.5 kDa MMP-3 indicates that the catalytic domain of MMP-3 is involved in plasmin(ogen) binding.

DISCUSSION

Antiangiogenic activity of angiostatin was first described in a plasminogen fragment consisting of the first four kringle domains (*8*) and later confirmed in an elastase-derived fragment (LBS I) containing kringles 1−3 (*8, 9*). Recently it was, however, reported that the isolated kringle 5 fragment of plasminogen displays a much more potent inhibition of microvascular endothelial cell migration than angiostatin (*25*). Macrophage-derived metalloelastase was shown to produce angiostatin from plasminogen and may contribute to angiostatin generation in the murine Lewis lung carcinoma model (*26*). Angiostatin-generating activity is present in tumor-bearing animals and serum-free conditioned medium of human prostate carcinoma cells (PC-3) (*27*). Two components released by PC-3 cells were identified, the plasminogen activator urokinase and free sulfhydryl donors, that are involved in angiostatin generation (*28*). Angiostatin may be generated by plasmin autodigestion in a reaction catalyzed by a protein disulfide isomerase or the presence of a free sulfhydryl donor (*28, 29*). Thus, the exact structural requirements for angiostatin activity as well as the mechanism(s) involved in its generation are not definitively established.

We have observed that an angiostatin-like fragment (containing kringles 1−4) can be generated from plasminogen with stromelysin-1 (MMP-3). MMP-3 indeed hydrolyzes the Glu^{59}−Asn^{60}, Pro^{447}−Val^{448}, and Pro^{544}−Ser^{545} peptide bonds in plasminogen, yielding a 55 kDa NH_2-terminal fragment comprising kringles 1−4, a 14 kDa domain corresponding to kringle 5, and a 30 kDa domain comprising the serine proteinase domain. The reaction is abolished in the presence of MMP inhibitors. MMP-3 has a broad substrate specificity (*30*), and it was previously reported to hydrolyze peptide bonds with Pro in the P1 position, e.g., Pro−Met in α_1-protease inhibitor (*31*) and Pro−Leu in human fibronectin (*32*).

Very recently, it was reported that MMP-7 (matrilysin) and MMP-9 (gelatinase B) also generate angiostatin-like fragments comprising kringles 1−4 from human plasminogen (*33*). Both MMP-7 and MMP-9 hydrolyze the Lys^{77}−Lys^{78} peptide bond, thus releasing the preactivation peptide. MMP-7 further cleaves the Pro^{447}−Val^{448} peptide bond, whereas MMP-9 hydrolyzes the Pro^{446}−Pro^{447} peptide bond. At an enzyme/substrate ratio of 1/15, MMP-7 hydrolyzes plasminogen faster than MMP-9 (within approximately 20 h, as compared to ≥100 h). MMP-3 at an enzyme/substrate ratio of 1/10 hydrolyzes the susceptible peptide bonds in plasminogen for more than 50% within 30 min. Because of potential differences of specific activities of MMP preparations, direct comparison of proteolytic activities toward plasminogen may, however, be difficult. Interestingly, MMP-3 generates concomitantly with the kringle 1−4 domain also the kringle 5 fragment, which was recently reported to have a strong antiangiogenic potency (*25*).

Different members of the MMP family may thus generate angiostatin-like fragments with different efficiency and with different composition. In addition, the contribution of MMP-3 and other MMP's to angiostatin generation in vivo will depend on the rate of their inactivation by endogenous inhibitors. This mechanism may be further complicated by interactions between different MMP's. Thus, proMMP-9 can be activated by MMP-3 (*34, 35*) and by MMP-7 (*36*). It remains to be shown whether and to what extent different MMP's contribute to angiostatin generation and to regulation of angiogenesis in vivo.

ACKNOWLEDGMENT

NH_2-terminal amino acid sequence analysis was performed by courtesy of Dr. F. Van Leuven (Center for Human Genetics, University of Leuven, Belgium). Skillful technical assistance by L. Frederix and B. Van Hoef is gratefully acknowledged.

REFERENCES

1. Sottrup-Jensen, L., Petersen, T. E., and Magnusson, S. (1978) *Atlas of Protein Sequences* (Dayhoff, M. O., Ed.) p 91, National Biomedical Research Foundation, Washington, DC.

2. Forsgren, M., Raden, B., Israelsson, M., Larsson, K., and Heden, L. O. (1987) *FEBS Lett. 213*, 254−260.
3. Robbins, K. C., Summaria, L., Hsieh, B., and Shah, R. J. (1967) *J. Biol. Chem. 242*, 2333−2342.
4. Wallen, P., and Wiman, B. (1970) *Biochim. Biophys. Acta 221*, 20−30.
5. Wallen, P., and Wiman, B. (1972) *Biochim. Biophys. Acta 257*, 122−134.
6. Collen, D. (1980) *Thromb. Haemostasis 43*, 77−89.
7. Lijnen, H. R., Bachmann, F., Collen, D., Ellis, V., Pannekoek, H., Rijken, D. C., and Thorsen, S. (1994) *J. Intern. Med. 236*, 415−424.
8. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. (1994) *Cell 79*, 315−328.
9. Cao, Y., Ji, R. W., Davidson, D., Schaller, J., Marti, D., Söhndel, S., McCance, S. G., O'Reilly, M. S., Llinas, M., and Folkman, J. (1996) *J. Biol. Chem. 271*, 29461−29467.
10. Sim, B. K. L., O'Reilly, M. S., Liang, H., Fortier, A. H., He, W., Madsen, J. W., Lapcevich, R., and Nacy, C. A. (1997) *Cancer Res. 57*, 1329−1334.
11. Wu, Z., O'Reilly, M. S., Folkman, J., and Shing, Y. (1997) *Biochem. Biophys. Res. Commun. 236*, 651−654.
12. O'Reilly, M. S., Holmgren, L., Chen, C., and Folkman, J. (1996) *Nat. Med. 2*, 689−692.
13. Matrisian, L. M. (1990) *Trends Genet. 6*, 121−125.
14. Woessner, J. F., Jr. (1991) *FASEB J. 5*, 2145−2154.
15. Birkedal-Hansen, H. (1995) *Curr. Opin. Cell Biol. 7*, 728−735.
16. Stetler-Stevenson, W. G., Aznavoorian, S., and Liotta, L. A. (1993) *Annu. Rev. Cell Biol. 9*, 541−573.
17. Lijnen, H. R., Van Hoef, B., and Collen, D. (1981) *Eur. J. Biochem. 120*, 149−154.
18. Lijnen, H. R., Van Hoef, B., Nelles, L., and Collen, D. (1990) *J. Biol. Chem. 265*, 5232−5236.
19. Suzuki, K., Enghild, J. J., Morodomi, T., Salvesen, G., and Nagase, H. (1990) *Biochemistry 29*, 10261−10270.
20. Nagase, H., Suzuki, K., Enghild, J. J., and Salvesen, G. (1991) *Biomed. Biochim. Acta 50*, 749−754.
21. Holvoet, P., Lijnen, H. R., and Collen, D. (1985) *J. Biol. Chem. 260*, 12106−12111.
22. Lijnen, H. R., Lasters, I., Verstreken, M., Collen, D., and Jespers, L. (1997) *Anal. Biochem. 248*, 211−215.
23. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350−4354.
24. Lijnen, H. R., De Cock, F., and Collen, D. (1994) *Eur. J. Biochem. 224*, 567−574.
25. Davidson, D. J., Reininger, I., Ennis, B. W., Quan, N., Haskell, C., Wang, J., and Henkin, J. (1997) Sixth International Workshop on the Molecular and Cellular Biology of Plasminogen Activation, San Diego, Abstract 200.
26. Dong, Z., Kumar, R., Yang, X., and Fidler, I. J. (1997) *Cell 88*, 801−810.
27. Gately, S., Twardowski, P., Stack, M. S., Patrick, M., Boggio, L., Cundiff, D. L., Schnaper, H. W., Madison, L., Volpert, O., Bouck, N., Enghild, J., Kwaan, H. C., and Soff, G. A. (1996) *Cancer Res. 56*, 4887−4890.
28. Gately, S., Twardowski, P., Stack, M. S., Cundiff, D. L., Grella, D., Castellino, F. J., Enghild, J., Kwaan, H. C., Lee, F., Kramer, R. A., Volpert, O., Bouck, N., and Soff, G. A. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 10868−10872.
29. Stathakis, P., Fitzgerald, M., Matthias, L. J., Chesterman, C. N., and Hogg, P. J. (1997) *J. Biol. Chem. 272*, 20641−20645.
30. Nagase, H., Fields, C. G., and Fields, G. B. (1994) *J. Biol. Chem. 269*, 20952−20957.
31. Mast, A. E., Enghild, J. J., Nagase, H., Suzuki, K., Pizzo, S. V., and Salvesen, G. (1991) *J. Biol. Chem. 266*, 15810−15816.
32. Wilhelm, S. M., Shao, Z. H., Housley, T. J., Seperack, P. K., Baumann, A. P., Gunja Smith, Z., and Woessner, J. F., Jr. (1993) *J. Biol. Chem. 268*, 21906−21913.
33. Patterson, B. C., and Sang, Q. A. (1997) *J. Biol. Chem. 272*, 28823−28825.
34. Okada, Y., Gonoji, Y., Naka, K., Tomita, K., Nakanishi, I., Iwata, K., Yamashita, K., and Hayakawa, T. (1992) *J. Biol. Chem. 267*, 21712−21719.
35. Ogata, Y., Enghild, J. J., and Nagase, H. (1992) *J. Biol. Chem. 267*, 3581−3584.
36. Imai, K., Yokohama, Y., Nakanishi, I., Ohuchi, E., Fujii, Y., Nakai, N., and Okada, Y. (1995) *J. Biol. Chem. 270*, 6691−6697.

BI9731798