

## Identification of the Proteolytic Enzyme Which Cleaves Human p75 TNF Receptor *in Vitro*

Koichi Katsura,\*† Minha Park,\* Maki Gatanaga,\* Ellen C. Yu,\* Kunio Takishima,†  
Gale A. Granger,\* and Tetsuya Gatanaga\*<sup>1</sup>

\*Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92717-3900; and †Department of Biochemistry I, National Defense Medical College, Tokorozawa, Saitama 359, Japan

Received April 3, 1996

The extracellular domains of the human 55 and 75 kD TNF receptors (p55 and p75 TNF-R) are proteolytically cleaved to produce 30 and 40 kD soluble fragments, respectively. In this study, the enzymatic activity involved in the cleavage of human p75 TNF-R, named TNF-R releasing enzyme (TRRE), was identified in the culture supernatant of PMA-stimulated THP-1 cells using an activity assay system established by our group. When THP-1 cells were stimulated with PMA, TRRE was released rapidly into the supernatant, reaching maximal activity within 3 hours. The release of TRRE into the culture supernatant depended on the concentration of PMA and FCS. TRRE activity was partially inhibited by chelating agents, suggesting that TRRE may be a metalloprotease-like enzyme. This is the first successful attempt to establish a stable TRRE source with a reliable assay system. © 1996 Academic Press, Inc.

Human tumor necrosis factor (TNF, TNF- $\alpha$ ) and lymphotoxin (LT, TNF- $\beta$ ) specifically bind to two related but distinct membrane receptors (TNF-R) of 55 and 75 kD with equally high affinity (1). Human soluble TNF-R's (sTNF-R's) or TNF binding proteins (TNF-BP's) are 30 and 40 kD fragments produced from the N-terminal extracellular domains of the p55 and p75 TNF-R's respectively (2–6). After being secreted, these soluble TNF-R's bind to TNF, thus regulating the cytokine's biological activities (7). Many cytokine receptors have soluble counterparts, such as interleukin-1 receptor (IL-1R) (8), IL-2R (9), IL-4R (10), IL-5R (11), IL-6R (12), IL-7R (13), IL-9R (14), colony stimulating factor receptor (CSF-R) (15) and nerve growth factor receptor (NGF-R) (16). This suggests the existence of a soluble counterpart of a membrane bound cytokine receptor is a common phenomenon, presumably playing important functions in the regulation of corresponding cytokine activities. Recently it has been suggested that metalloproteases are involved in the cleavage of several membrane receptors and antigens (17–19), including both the p55 and p75 TNF-R's (17,20,21), and the membrane bound pro-TNF- $\alpha$  (20,22–24). We have previously shown that human monocytic THP-1 cells stimulated with phorbol 12-myristate 13-acetate (PMA) produced both soluble p55 and p75 TNF-R by proteolytic cleavage rather than by alternative splicing of mRNA (25,26). Furthermore, enzyme activity involved in the cleavage of TNF-R was detected in the culture supernatant of PMA-stimulated THP-1 cells (26), although existence of a membrane-bound enzyme has not been dismissed. We have developed a stable enzyme source of the proteolytic activity resulting in the generation of soluble TNF-R as well as a reliable *in vitro* activity assay system. We name this proteolytic activity, TNF-R releasing enzyme (TRRE), which is secreted into the culture supernatant by PMA-stimulated THP-1 cells. In the present study, we employ our assay system to identify and describe TRRE activity, which may be caused by a metalloprotease-like enzyme.

### MATERIALS AND METHODS

*TRRE induction from PMA-stimulated THP-1 cells.* THP-1 cells (American Type Culture Collection, Rockville, MD) at a density of  $1 \times 10^6$  cells/ml in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) with 1% heat-inactivated FCS (Irvine

<sup>1</sup> Correspondence author: Fax: (714) 824-5048. E-mail: tgatanag@uci.edu.

Scientific, Santa Ana, CA) were stimulated with  $10^{-6}$  M PMA (Sigma Chemical, St. Louis, MO) for 30 min in 5%  $\text{CO}_2$  at  $37^\circ\text{C}$ . The cells were washed once with serum-free RPMI 1640 to remove PMA, resuspended in the same volume of RPMI 1640 with 1% FCS, and then incubated for 2 more hours. The supernatant collected was the enzyme source of later experiments.

*Construction of COS-1 cells transduced with human p75 TNF-R cDNA.* The human p75 TNF-R cDNA was transduced into COS-1 cells (American Type Culture Collection). COS-1 cells do not express either p55 or p75 TNF-R. Human p75 TNF-R cDNA was cloned from human monocytic U937 cDNA library in  $\lambda\text{gt}10$  (Clontech Laboratories, Palo Alto, CA) and was inserted into the EcoRI site of the mammalian expression vector pcDNA3, (Invitrogen, San Diego, CA) which contains the neomycin resistance gene. This construct was transfected into COS-1 by calcium phosphate-DNA precipitation method described by Chen and Okayama (27). These transfected cells were then cultured in RPMI 1640 supplemented with 600  $\mu\text{g}/\text{ml}$  Geneticin (G418) (GIBCO BRL Life Technologies, Gaithersburg, MD) and 10% FCS for selection of neomycin resistant cells. These transfected cells expressed approximately 70,000 receptors/cell by Scatchard analysis (data not shown), which is about 50 times the level expressed on most human cells. These cells were termed C75R and used as the substrate source for the TRRE assay.

*Establishment of a reliable assay for detection and quantification of TRRE activity.* C75R cells and COS-1 cells were seeded into 24 well cell culture plates at a density of  $2.5 \times 10^5$  cells/well and incubated overnight in 5%  $\text{CO}_2$  at  $37^\circ\text{C}$ . After aspirating the medium in the wells, 300  $\mu\text{l}$  of the samples with TRRE activity in question were incubated in the wells of both C75R and COS-1 for 30 min at  $37^\circ\text{C}$ . The supernatants were collected and assayed for concentration of soluble p75 TNF-R by ELISA (26).

TRRE activity is calculated by the following formula:

A: Amount of soluble p75 TNF-R in the C75R plate treated with TRRE sample.

B: Amount of soluble p75 TNF-R spontaneously released from C75R plate treated with only medium or buffer without exogenous TRRE.

C: Amount of soluble p75 TNF-R from COS-1 plate treated with TRRE sample or the back ground level of soluble p75 TNF-R released by THP-1; there is significant degradation of soluble p75 TNF-R during 30 min incubation.

Net release of soluble p75 TNF-R due to TRRE activity = A - B - C

The net release was considered to be the amount of TRRE activity and 1 unit (U) of TRRE activity was defined as 1 pg of soluble p75 TNF-R net release from  $2.5 \times 10^5$  C75R cells in 30 min.

## RESULTS AND DISCUSSION

*Cell line and PMA dependence.* THP-1 cells and human monocytes at a density of  $1 \times 10^6$  cells/ml RPMI 1640 with 1% FCS were stimulated with  $10^{-7}$  and  $10^{-6}$  M PMA for 30 min in 5%  $\text{CO}_2$  at  $37^\circ\text{C}$ . The stimulated cells were washed with medium and resuspended in the same volume of PMA-free medium with 1% FCS for two more hours in 5%  $\text{CO}_2$  at  $37^\circ\text{C}$ .  $10^{-6}$  M PMA had an efficiently strong stimulation of THP-1 cells and human monocytes, inducing a TRRE release at concentrations of 1304 and 883 U/ml, respectively (Table 1),  $10^{-7}$  M PMA stimulation induced relatively low amounts of TRRE and  $10^{-8}$  M PMA stimulation did not induce any TRRE from either monocytes or THP-1 cells (data not shown). PMA stimulation at the concentration of  $10^{-6}$  M was adopted in all subsequent experiments for the induction of TRRE from THP-1 cells. This data shows that the procedures applied for the induction of TRRE and its assay system are effective not only for a continuous tumor cell line such as THP-1, but also for normal human monocytes. Thus, TRRE activity is probably not a unique characteristic of a transformed cell line, but a common trait of human monocytes obtained from peripheral blood. The reason why a PMA concentration as high as  $10^{-6}$  M was required for an effective induction of TRRE may be because we used a pulse stimulation of 30 min for the cells, followed by a wash and incubation in PMA-free media for two more hours. Also, it is quite possible that cells release high levels of active TRRE

TABLE 1  
Induction of TRRE from Human Monocytes and THP-1 Cells

Cell	Activator	TRRE Activity (U/ml)
Human monocyte	PMA $10^{-7}$ M	51
	$10^{-6}$ M	883
THP-1	PMA $10^{-7}$ M	368
	$10^{-6}$ M	1304

TABLE 2  
Stability of TRRE Activity

Temperature	% control of TRRE activity	pH	% control of TRRE activity
4°C for 48 hours	82	6.0	52
56°C for 30 min.	84	7.0	100
100°C for 15 min.	16	8.0	69
		9.0	73

immediately when stimulated with PMA during the 30 min pulse stimulation and that TRRE released during this period is discarded when the cells are washed and incubated with fresh medium.

*Physical condition of TRRE activity.* TRRE activity was stable when stored at  $-70^{\circ}\text{C}$ . However, TRRE bioactivity was reduced by incubation at  $4^{\circ}\text{C}$  for 2 days, heating at  $56^{\circ}\text{C}$  for 30 min, and boiling for 15 min to 82%, 84% and 16% of its initial activity, respectively. TRRE supernatants at various pH levels were pre-incubated at  $37^{\circ}\text{C}$  for 30 min and then applied to TRRE assay after adjusting the pH of all supernatants to 7.0. TRRE samples pre-incubated at pH 6.0, 7.0, 8.0, and 9.0 showed 52%, 100%, 69%, and 73% of TRRE activity contained in the original TRRE supernatant (pH 7.4), respectively (Table 2).

*FCS dependence.* THP-1 cells were stimulated with  $10^{-6}$  M PMA in RPMI 1640 with 1% FCS for 30 min. The stimulated cells were then washed and incubated for 2 more hours in PMA-free RPMI 1640 with 0%, 1%, and 10% FCS, which led to the release of 224, 1356, and 2275 U/ml TRRE, respectively (Table 3). This suggests that some serum factors are required by cells for a normal response to PMA. However, FCS-containing medium alone in absence of PMA-stimulation did not induce TRRE activity (data not shown). Since incubation of PMA-stimulated THP-1 cells in the presence of 1% FCS would significantly decrease the level of contaminating proteins from FCS and increase the specific activity of TRRE in the supernatant, TRRE induction at 1% FCS concentration was adopted for subsequent experiments.

*TRRE kinetics.* THP-1 cells were stimulated with  $10^{-6}$  M PMA in RPMI 1640 with 1% FCS for 30 min and then washed and resuspended in the same volume of PMA-free medium with 1% FCS. The cells were then incubated for another 2 to 23 hours making the total induction time of TRRE 3 to 24 hours from initial stimulation. This kinetic study revealed that the release of TRRE into culture supernatants peaked as early as 3 hours, followed by a gradual decline afterwards, whereas the level of sTNF-R derived from THP-1 cells increased over time (Fig. 1). Consequently, in order to obtain higher TRRE activity with lower sTNF-R background, 2 hours incubation (total 3 hours induction) was adopted in subsequent experiments. The rapid release of TRRE from THP-1 cells in response to PMA-stimulation suggests a reservoir of TRRE stored in the cytoplasm or on the cell surface which can be quickly released in response to an exogenous stimulant.

*Dilution vs activity.* TRRE supernatants produced as described were serially diluted up to 1:256 dilution. Detectable levels of TRRE activity were present in samples diluted to 16 fold (Fig. 2). While the level of sTNF-R present in TRRE supernatants decreased in proportion to their dilution, no significant differences in TRRE activity were found between the original and 2 times diluted

TABLE 3  
Effect of FCS Concentration on the Induction of TRRE

FCS concentration	TRRE activity (U/ml)
0%	224
1%	1356
10%	2275

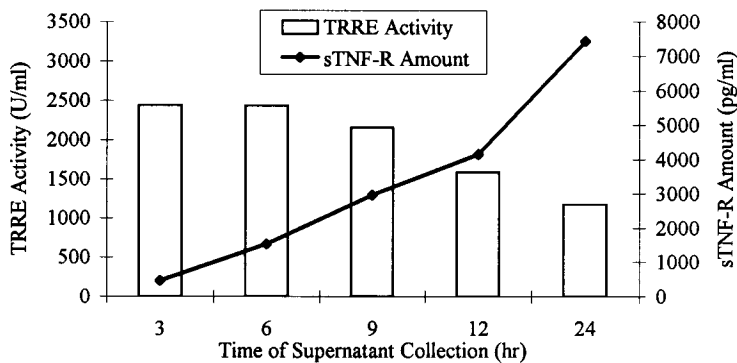


FIG. 1. Kinetics of TRRE activity and sTNF-R release was conducted on the incubation time from 3 to 24 hours including initial 30 min PMA-stimulation. TRRE activity: bar graph; sTNF-R amount: line graph

supernatant, suggesting a depletion of substrates and that the level of TRRE activity in the original supernatant might be saturating our assay system beyond its capacity.

*Classification of TRRE.* The effects of various protease inhibitors on TRRE activity were investigated in order to classify this enzyme. TRRE supernatant was assayed for activity and then diluted to 1000 U/ml of net activity. This 1000 U/ml TRRE was preincubated with and without protease inhibitors at 37°C for 30 min and then assayed for TRRE activity. The level of activity measured in the absence of any inhibitor was taken as a control, and the level of activity in samples of TRRE coincubated with various inhibitors were expressed as percent activity relative to the control. No significant inhibition was found with phenylmethylsulfonyl fluoride (PMSF) and 3,4-dichloroisocoumarin (3,4-DCI) (serine protease inhibitors), and N-tosyl-L-lysine chloromethyl ketone (TLCK) (serine and cysteine protease inhibitor). However, partial inhibition was obtained with chelating agents, such as 1,10-phenanthroline, ethylenediaminetetraacetic acid (EDTA), and ethylene glycol-bis(2-aminoethyl ether) tetraacetic acid (EGTA) (Table 4). Since chelating agents are potent inhibitors of metalloproteases, these results indicate that TRRE is a metalloprotease-like enzyme. This data is compatible with two reports that showed the involvement of metalloprotease(s) in the production of soluble TNF-R by use of a specific metalloprotease inhibitor (TNF- $\alpha$  protease inhibitor; TAPI) (17,21). However, this is the first report that has specifically identified the enzyme activity involved in the cleavage of TNF-R using our newly established assay system.

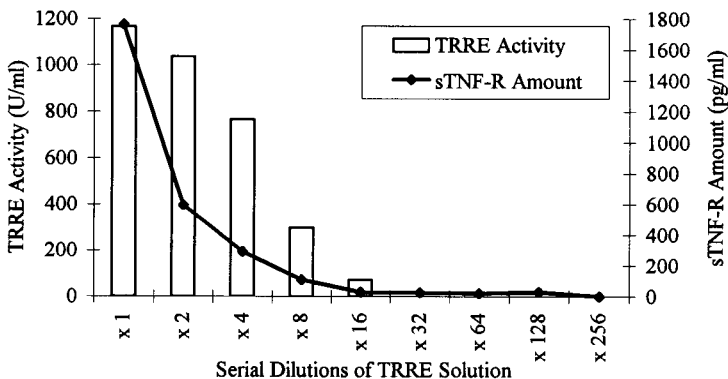


FIG. 2. TRRE contained in the supernatant(s) were serially diluted with 1% FCS-contained RPMI. TRRE activity: bar graph; sTNF-R amount: line graph

TABLE 4  
Effect of Various Protease Inhibitors on TRRE Activity

Inhibitor		% control (mean $\pm$ S.E.)
PMSF	2 mM	106 $\pm$ 4
TLCK	0.1 mM	99 $\pm$ 16
3,4-DCI	0.1 mM	93 $\pm$ 10
EDTA	1 mM	72 $\pm$ 9
EGTA	1 mM	74 $\pm$ 8
1, 10-phenanthroline	4 mM	35 $\pm$ 11

## REFERENCES

- Holtmann, H., and Wallach, D. (1987) *J. Immunol.* **139**, 1161–1167.
- Seckinger, P., Isaaz, S., and Dayer, J. M. (1989) *J. Biol. Chem.* **264**, 11966–11973.
- Peetre, C., Thysell, H., Grubb, A., and Olsson, I. (1988) *Eur. J. Haematol.* **41**, 414–419.
- Olsson, I., Lantz, M., Nilsson, E., Peetre, C., Thysell, H., Grubb, A., and Adolf, G. (1989) *Eur. J. Haematol.* **42**, 270–275.
- Gatanaga, T., Lentz, R., Masunaka, I., Tomich, J., Jeffes, E. W., Baird, M., and Granger, G. A. (1990) *Lymphokine Res.* **9**, 225–229.
- Gatanaga, T., Hwang, C. D., Kohr, W., Cappuccini, F., Lucci, J. A., Jeffes, E. W., Lentz, R., Tomich, J., Yamamoto, R. S., and Granger, G. A. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8781–8784.
- Tartaglia, L. A., and Goeddel, D. V. (1992) *Immunol. Today* **13**, 151–153.
- Colotta, F., Re, F., Muzio, M., Bertini, R., Polentarutti, N., Sironi, M., Giri, J. G., Dower, S. K., Sims, J. E., and Mantovani, A. (1993) *Science* **261**, 472–475.
- Baran, D., Korner, M., and Theze, J. (1988) *J. Immunol.* **141**, 539–546.
- Mosley, B., Beckmann, M. P., March, C. J., Idzerda, R. L., Gimpel, S. D., VandenBos, T., Friend, D., Alpert, A., Anderson, D., and Jackson, J. (1989) *Cell* **59**, 335–348.
- Takaki, S., Tominaga, A., Hitoshi, Y., Mita, S., Sonoda, E., Yamaguchi, N., and Takatsu, K. (1990) *EMBO J.* **9**, 4367–4374.
- Novick, D., Engelmann, H., Wallach, D., and Rubinstein, M. (1989) *J. Exp. Med.* **170**, 1409–1414.
- Goodwin, R. G., Friend, D., Ziegler, S. F., Jerzy, R., Falk, B. A., Gimpel, S., Cosman, D., Dower, S. K., March, C. J., and Namen, A. E. (1990) *Cell* **60**, 941–951.
- Renauld, J. C., Druetz, C., Kermouni, A., Houssiau, F., Uyttenhove, C., Van Roost, E., and Van Snick, J. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5690–5694.
- Fukunaga, R., Seto, Y., Mizushima, S., and Nagata, S. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8702–8706.
- Zupan, A. A., Osborne, P. A., Smith, C. E., Siegel, N. R., Leimgruber, R. M., and Johnson, E. M. J. (1989) *J. Biol. Chem.* **264**, 11714–11720.
- Müllberg, J., Durie, F. H., Otten-Evans, C., Alderson, M. R., Rose-John, S., Cosman, D., Black, R. A., and Mohler, K. M. (1995) *J. Immunol.* **155**, 5198–5205.
- DiStefano, P. S., Chelsea, D. M., Schick, C. M., and McKelvey, J. F. (1993) *J. Neurosci.* **13**, 2405–2414.
- Leca, G., Mansur, S. E., and Bensussan, A. (1995) *J. Immunol.* **154**, 1069–1077.
- Crowe, P. D., Walter, B. N., Mohler, K. M., Otten-Evans, C., Black, R. A., and Ware, C. F. (1995) *J. Exp. Med.* **181**, 1205–1210.
- Björnberg, F., Lantz, M., and Gullberg, U. (1995) *Scand. J. Immunol.* **42**, 418–424.
- Mohler, K. M., Sleath, P. R., Fitzner, J. N., Cerretti, D. P., Alderson, M., Kerwar, S. S., Torrance, D. S., Otten-Evans, C., Greenstreet, T., and Weerawarna, K. (1994) *Nature* **370**, 218–220.
- Gearing, A. J., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, A. H., Drummond, A. H., Galloway, W. A., Gilbert, R., and Gordon, J. L. (1994) *Nature* **370**, 555–557.
- McGeehan, G. M., Becherer, J. D., Bast, R. C. J., Boyer, C. M., Champion, B., Connolly, K. M., Conway, J. G., Furdon, P., Karp, S., and Kidao, S. (1994) *Nature* **370**, 558–561.
- Gatanaga, T., Hwang, C. D., Gatanaga, M., Cappuccini, F., Yamamoto, R. S., and Granger, G. A. (1991) *Cell Immunol.* **138**, 1–10.
- Hwang, C., Gatanaga, M., Granger, G. A., and Gatanaga, T. (1993) *J. Immunol.* **151**, 5631–5638.
- Chen, C. A., and Okayama, H. (1988) *Biotechniques* **6**, 632–638.