Fractalkine (FK, CX3CL1) is a novel multidomain protein expressed on the surface of endothelial cells. As a full-length transmembrane protein, FK binds cells expressing CX3CR1, its cognate receptor, with high affinity. Proteolytic cleavage of FK releases a soluble form that is a potent chemoattractant for monocytes, T cells, and natural killer cells. Activation of protein kinase C dramatically increases the rate of this cleavage. Regulation of FK cleavage is critical for maintaining the balance between the immobilized and soluble forms, but the protease responsible has not been identified. Here we report that tumor necrosis factor-α-converting enzyme (TACE) is primarily responsible for the inducible cleavage of FK. After transfection into host cells, the proteolytic cleavage of FK was blocked by TACE-specific inhibitors and was not detected in cells genetically altered to remove TACE activity. In contrast, the constitutive cleavage of FK was not mediated by TACE and proceeded normally in TACE-null fibroblasts. We conclude that TACE is primarily responsible for the inducible cleavage of FK. These studies identify a potentially important link between local generation of potent cytokines and control of the balance between the cell adhesion and chemotactic properties of FK.

Fractalkine (FK) is a structurally unusual protein in which a chemokine-like domain is located atop a mucin stalk connected to a transmembrane domain (1). FK is expressed on the surface of endothelial cells (2), neurons (3), and epithelial cells (4), and we and others have shown that full-length FK can efficiently capture cells expressing CX3CR1, its cognate receptor. A soluble form of FK has also been described and is thought to be produced by cleavage at a di-arginine sequence (1, 3) next to the transmembrane domain. Incubation of cells with phorbol to be produced by cleavage at a di-arginine sequence (1, 3) next to the transmembrane domain. FK is expressed on the surface of endothelial cells (2), neurons (3), and epithelial cells (4), and natural killer cells. Activation of protein kinase C dramatically increases the rate of this cleavage. Regulation of FK cleavage is critical for maintaining the balance between the immobilized and soluble forms, but the protease responsible has not been identified. Here we report that tumor necrosis factor-α-converting enzyme (TACE) is primarily responsible for the inducible cleavage of FK. After transfection into host cells, the proteolytic cleavage of FK was blocked by TACE-specific inhibitors and was not detected in cells genetically altered to remove TACE activity. In contrast, the constitutive cleavage of FK was not mediated by TACE and proceeded normally in TACE-null fibroblasts. We conclude that TACE is primarily responsible for the inducible cleavage of FK. These studies identify a potentially important link between local generation of potent cytokines and control of the balance between the cell adhesion and chemotactic properties of FK.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human soluble FK and a biotinylated polyclonal anti-FK antibody were obtained from R & D Systems (Minneapolis, MN). Rabbit anti-human FK antibody was from Torrey Pines Bioslabs (San Diego, CA). TAPI-2 (IC-3) was a generous gift from Drs. Douglas Cerretti and Roy Black (Immunex, Seattle, WA). GM6001 was from Chemicon (Temecula, CA). PMA and 3,4-dichloroisocoumarin were from Calbiochem. Dicycleglycerol (1,2-dioctanoyl-sn-glycerol (8:0)) was from Biomol (Plymouth Meeting, PA). Fetal calf serum was from Hyclone Laboratories (Logan, UT). Geneticin was from Life Technologies, Inc. All other chemicals were from Sigma.

Recombinant Cell Lines—ECV cells expressing full-length FK (7) were a generous gift from Drs. Daniel Dairaghi and Thomas J. Schall (ChemoCentryx, San Carlos, CA) and were maintained in minimal essential medium and Earle’s balanced salt solution supplemented with 10% fetal calf serum and antibiotics. ECV cells were also transfected with full-length FK in which the FLAG epitope (GSYKDDDDK) was inserted between the chemokine domain and the mucin stalk or with a mutated form of FK in which the arginines at positions 347 and 348 were changed to alanines. All cDNA constructs were made in pcDNA3 (Invitrogen, Carlsbad, CA), and all transfections were done with LipofectAMINE (Life Technologies), following the manufacturer’s instructions. Chinese hamster ovary (CHO)-M1 (13) cells, which do not process TACE to an active form, were a generous gift from Dr. Joan Massague (Memorial Sloan-Kettering Hospital, New York, NY). These cells express a neomycin resistance gene, and transfectants were produced by co-transfecting full-length FK and a zoeinic resistance gene, pZeoSV2. These cells were maintained in minimal essential medium and Earle’s balanced salt solution supplemented with 10% fetal calf serum and penicillin and streptomycin. Geneticin (800 μg/ml), and zeocin (400 μg/ml; Invitrogen). Stably transfected cell lines were selected by flow
cytometry by staining the cells with biotinylated polyclonal anti-FK antibody (1.5 μg/ml; R & D Systems), followed by streptavidin-phycocerythrin (1 μg/ml; Pharmingen, San Diego, CA). Wild-type CHO cells stably expressing FK were also prepared. TACE−/− and TACE+/+ fibroblasts were generous gifts from Drs. Douglas Cerretti and Roy Black. These cells were maintained in Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium supplemented with 1% fetal calf serum and antibiotics. These cells were maintained in Dulbecco’s medium and Earle’s balanced salt solution; 20 min before the addition of PMA, metalloprotease inhibitors (or control vehicle) were added. After the conditioned medium was collected, the cells were lysed by adding immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM Na pyrophosphate, and 1% Triton X-100) supplemented with protease inhibitors (1 μl leupeptin, 1 mM phenylmethylsulfonyl fluoride, 2.1 μg/ml aprotinin, and 1 μg/ml peptatin). The cell lysates were precleared by incubation with 100 μl of anti-rabbit IgG-agarose (Sigma) that had been blocked by preincubation with 2% (w/v) bovine serum albumin in immunoprecipitation buffer for 2 h at 4 °C. The samples were spun for 10 min in a microfuge, and FK was immunoprecipitated by incubation with rabbit anti-human FK antibody (6 μg/ml; Torrey Pines) overnight at 4 °C. Anti-rabbit IgG-agarose (100 μl, preblocked with 2% (w/v) bovine serum albumin) was used to precipitate the FK-antibody complex. Samples were resuspended in SDS sample buffer, incubated for 10 min without heating, and electrophoresed as described above.

**RESULTS**

To study the cleavage of FK, we used cell lines that stably expressed either wild-type or mutated forms of full-length FK. Each was expressed at the cell surface at similar levels, as determined by flow cytometry (data not shown). Soluble FK was detected in the conditioned medium of transfected epithelial cell lines by Western blot, as described under “Experimental Procedures,” and recombination full-length FK (50 ng) was run as a molecular weight indicator. A, PMA-induced cleavage was blocked by EDTA (5 mM). B, mutation of the di-arginine sequence in FK (RR-FK) did not prevent PMA-induced cleavage. Data shown are the mean of three independent experiments. Error bars represent S.E.M. DMSO, Me2SO; WT, wild-type.
Fractalkine cleavage by TACE

Fractalkine, and its concentration was markedly increased by the addition of PMA for 30 min (Fig. 1A). Inclusion of EDTA in the medium reduced cleavage, indicating that the protease activity depends on divalent cations. The di-arginine sequence in FK located in juxtaposition to the transmembrane domain has been implicated as the cleavage site. To examine the importance of this sequence in the proteolysis of FK, we mutated these two arginines to alanines. This mutation had no effect on the PMA-induced cleavage (Fig. 1B).

Metalloproteases are divalent cation-dependent enzymes and are known to cleave membrane proteins. We therefore asked whether cleavage of FK was mediated by a metalloprotease. The metalloprotease inhibitor 1,10-phenanthroline (14) partially blocked FK cleavage, and the effect was dose-dependent (Fig. 2A); additional inhibition was not seen at higher concentrations (data not shown). Similar results were obtained with GM6001 (15), a metalloprotease inhibitor of broad specificity (Fig. 2B). TAPI-2 is a hyroxamate-based inhibitor of matrix metalloproteases that has high activity against TACE, the protease that cleaves TNF-α (13, 16). TAPI-2 blocked PMA-induced FK cleavage effectively and in a dose-dependent manner (Fig. 3). These data suggested that the enzyme that cleaved FK is very similar or possibly identical to TACE.

We next examined FK cleavage in CHO-M1 cells, a cell line in which known substrates of TACE are not shed (13, 17). After transfection with full-length FK, we noted higher levels of soluble FK in the conditioned medium of wild-type CHO cells than in the medium of transfected ECV304 cells (Figs. 1–3), suggesting a higher rate of constitutive cleavage (Fig. 4A). Addition of PMA induced further cleavage of FK in both wild-type CHO and CHO-M1 cells but at a markedly lower level in the CHO-M1 cells (Fig. 4A). Addition of TAPI-2 did not affect the amount of soluble FK in the medium, confirming the absence of TACE activity in the CHO-M1 cells (data not shown).

Western blots of the cell lysates confirmed that FK was expressed at comparable levels in the CHO wild-type and CHO-M1 cell lines (Fig. 4B).

To determine whether TACE was responsible for the constitutive cleavage, we followed the accumulation of soluble FK in the conditioned medium of unactivated transfected CHO and CHO-M1 cells (Fig. 5). The rate of accumulation of soluble FK was similar in these two cell lines, particularly at the later time points. These data suggest that, unlike the PMA-induced cleavage of FK, TACE is not primarily responsible for the constitutive cleavage. Consistent with this result, TAPI-2 had little or no effect on the rate of constitutive cleavage of FK (data not shown).

To further test the hypothesis that TACE was the protease responsible for the induced cleavage of FK, we transfected TACE−/− fibroblasts with FK. Similar surface expression of FK was obtained in stably transfected TACE−/− and TACE+/+ fibroblasts (Fig. 6). Western blots of cell lysates also indicated very similar levels of FK expression in the wild-type and TACE−/− fibroblasts (data not shown). Addition of PMA re-
FIG. 6. Reduction in PMA-induced FK cleavage in TACE-null fibroblasts. Fibroblasts from TACE wild-type (TACE+/+) and TACE-deficient (TACE−/−) mice were transfected with FK, and the PMA-inducible cleavage was determined. A, FK was expressed at comparable levels in wild-type (FK/TACE+/+) and TACE-deficient (FK/TACE−/−) fibroblasts. B, soluble FK in the conditioned medium was measured after incubation with PMA. Untransfected fibroblasts (TACE−/−) are shown as a negative control for the Western blot. The broadness of the FK bands is attributable to heavy glycosylation by the fibroblast cell line. Data shown are representative of three experiments. DMSO, Me2SO.

sulted in robust cleavage of FK in the wild-type but not in the TACE−/− fibroblasts (Fig. 6, B and C).

**DISCUSSION**

Emerging in vivo data suggest an important role for FK in transplant rejection (18, 19) and susceptibility to coronary artery disease (20), but it is not clear whether this is attributable to full-length FK acting as an adhesion molecule, to soluble FK acting as a chemoattractant, or to both. Because regulation of the cleavage event that generates soluble FK is likely to have profound effects on the biological functions of FK, we sought to identify the responsible protease(s). The major finding in this paper is that TACE, a well-characterized protease that generates biologically active TNF-α, accounts for virtually all of the inducible cleavage of FK. In contrast, the constitutive cleavage of FK was not mediated by TACE and was not blocked by typical metalloprotease inhibitors. The di-arginine sequence of fractalkine at the plasma membrane interface was not required for either the inducible or the constitutive cleavage.

Several lines of evidence support the claim that TACE is responsible for the inducible cleavage of FK. First, cleavage was blocked by chelation of divalent cations with EDTA. Second, three structurally diverse metalloprotease inhibitors blocked cleavage. Third, there was little inducible cleavage detected when FK was expressed in CHO cells that had been genetically modified to prevent the generation of proteolytically active TACE. Fourth, little or no inducible cleavage of FK was evident in fibroblasts derived from TACE−/− mice. Taken together, these data provide strong evidence that virtually all of the inducible cleavage of FK is mediated by TACE. Interestingly, the constitutive cleavage of FK seen in both the ECV and CHO cells was also present in the TACE-null CHO-M1 and TACE−/− fibroblasts. These data indicate that TACE is not responsible for the constitutive cleavage of FK.

Several full-length membrane proteins are cleaved to generate soluble forms that retain biological activity. These include a number of different amino acid sequences. Consistent with this hypothesis, deletion of at least 12 amino acids was necessary to completely block shedding of TNF-α (24). The di-arginine sequence near the plasma membrane has been implicated as the cleavage site that generates soluble FK (1, 3), but the evidence for this has not been direct. In this study, we found that mutation of these two arginines to alanines had no effect on either the inducible or the constitutive cleavage of FK. These data suggest that extensive mutagene sis of the FK stalk just amino-terminal to the plasma membrane may be required to create a noncleavable form of FK.

Recently, Matloubian et al. (25) described CXCL16, a second member of the CX3C family of chemokines. CXCL16 is expressed on dendritic cells, and binds to naive CD8 T cells, natural killer T cells, and a subset of CD4 T cells (25). CXCL16 exists in both membrane-bound and soluble forms, and it is reasonable to speculate that soluble CXCL16 might attract T cells to the dendritic cells. This chemokine may therefore serve to facilitate interactions between dendritic cells and T cells.
The protease responsible for CXCL16 cleavage has not been characterized, but the findings presented here make TACE a likely candidate. Whether mice expressing a noncleavable form of CXCL16 would have an immune deficit remains to be determined.

In summary, we have found that TACE, the ADAMS family metalloprotease that cleaves TNF-α, is primarily responsible for the inducible cleavage of FK. The relative contributions of the full-length and soluble forms of FK in mediating cell-cell interactions are not known, but the identification of TACE as the cleavage enzyme should aid in this effort, as well as facilitate creation of a mouse with a noncleavable form of FK. This novel role for TACE identifies a potentially important link between local cytokine production and the cleavage of FK in activated cells.

Note—While this paper was in the review process, Garton et al. (26) reported very similar results.

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REFERENCES

1. Bazan, J. F., Bacon, K. B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D. R., Zlotnik, A., and Schall, T. J. (1997) Nature 385, 640–644
2. Harrison, J. K., Jiang, Y., Wees, E. A., Salafranca, M. N., Liang, H.-X., Feng, L., and Belardelli, L. (1999) J. Leukoc. Biol. 66, 907–944
3. Harrison, J. K., Jiang, Y., Chen, S., Xia, X., Maciejewski, D., McNamara, R. K., Streit, W. J., Salafranca, M. N., Adhikari, S., Thompson, D. A., Botti, P., Bacon, K. B., and Feng, L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10986–10991
4. Lucas, A. D., Chadwick, N., Warren, B. F., Jewell, D. P., Powrie, F., and Greaves, D. R. (2001) Am. J. Pathol. 158, 855–866
5. Pan, Y., Lloyd, C., Zhou, H., Delich, S., Deeds, J., Gonzalez, J.-A., Yath, J., Gosselin, M., Ma, J., Dussault, B., Woolf, E., Alperin, G., Culpepper, J., Gutierrez-Ramos, J. C., and Oettinger, D. (1997) Nature 387, 611–617
6. Muehlhofer, A., Saubermann, L. J., Gu, X., Luedtke-Heckenkamp, K., Xavier, R., Blumberg, R. S., Podolsky, D. K., MacDermott, R. P., and Reinecke, H.-C. (2000) J. Immunol. 164, 3368–3376
7. Imai, T., Hieshima, K., Haskell, C., Baba, M., Nagira, M., Nishimura, M., Kakizaki, M., Takagi, S., Nomiyama, H., Schall, T. J., and Yoshie, O. (1997) Cell 91, 521–530
8. Black, R. A., Rauch, C. T., Kozlowski, C. J., Peschon, J. J., Slack, J. L., Slack, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J., and Cerretti, D. P. (1997) Nature 385, 729–733
9. Peschon, J. J., Slack, J. L., Reddy, P., Stocking, K. L., Sunnarborg, S. W., Lee, D. C., Russell, W. E., Castner, B. J., Johnson, R. S., Fitzner, J. N., Boyce, R. W., Nelson, N., Kozlowski, C. J., Wolfson, M. F., Rauch, C. T., Cerretti, D. P., Paxton, R. J., March, C. J., and Black, R. A. (1998) Science 282, 1281–1284
10. Feehan, C., Darlak, K., Kahn, J., Walcheck, B., Spatola, A. F., and Kishimoto, T. K. (1996) J. Biol. Chem. 271, 7019–7024
11. Buxbaum, J. D., Liu, K.-N., Luo, Y., Slack, J. L., Stocking, K. L., Peschon, J. J., Johnson, R. S., Castner, B. J., Cerretti, D. P., and Black, R. A. (1998) J. Biol. Chem. 273, 27765–27767
12. Hooper, N. M., Karran, E. H., and Turner, A. J. (1997) Biochem. J. 321, 265–278
13. Arribas, J., Coodly, L., Vollmer, P., Kishimoto, T. K., Rose-John, S., and Massague, J. (1996) J. Biol. Chem. 271, 11376–11382
14. Dri, P., Gasparini, C., Menegazzi, R., Cramer, R., Alberi, L., Presani, G., Garbisa, S., and Patriarca, P. (2000) J. Immunol. 165, 2165–2172
15. Imai, T., Hieshima, K., Haskell, C., Baba, M., Nagira, M., Nishimura, M., Kakizaki, M., Takagi, S., Nomiyama, H., Schall, T. J., and Yoshie, O. (1997) Nature 385, 521–530
16. 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
17. Merlos-Suárez, A., Fernández-Larrea, J., Reddy, P., Baselga, J., and Arribas, J. (1998) J. Biol. Chem. 273, 24955–24962
18. Haskell, C. A., Hancock, W. W., Salant, D. J., Gao, W., Ceizmada, V., Peters, W., Faia, K., Fitouri, O., Rottman, J., and Charo, I. F. (2001) J. Clin. Invest. 108, 679–688
19. Robinson, L. A., Nataraj, C., Thomas, D. W., Howell, D. N., Griffiths, R., Bautch, V., Patel, D. D., Feng, L., and Coffman, T. M. (2000) J. Immunol. 165, 6067–6072
20. Meotti, D., Faure, S., Fumeron, F., Amaara, M. E. W., Seknadji, P., McDermott, D. H., Debé, P., Aumont, M. C., Murphy, P. M., de Prost, D., and Combadière, C. (2001) Blood 97, 1925–1928
21. Black, R. A., and White, J. M. (1998) Curr. Opin. Cell Biol. 10, 654–659
22. Althoff, K., Reddy, P., Volz, N., Rose-John, S., and Müllerberg, J. (2000) Eur. J. Biochem. 267, 2624–2631
23. SadhuBhak, R., Santhamman, K. R., Reddy, P., Peschon, J. J., Black, R. A., and Sen, L. (1999) J. Biol. Chem. 274, 10511–10516
24. Decoster, E., Vanhasebroeck, B., Vandenaebbele, P., Groten, J., and Fiers, W. (1995) J. Biol. Chem. 270, 18473–18478
25. Matloubian, M., David, A., Engel, S., Ryan, J. E., and Cyster, J. G. (2000) Nat. Immunol. 1, 298–304
26. Garton, K. J., Gough, P. J., Blobel, C. P., Murphy, G., Greaves, D. R., Dempsey, P. J., and Raines, E. W. (2001) J. Biol. Chem. 276, 37993–38001
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