Brief Definitive Report

A Metalloprotease Inhibitor Blocks Shedding of the 80-kD TNF Receptor and TNF Processing in T Lymphocytes

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Summary

TNF is synthesized as a 26-kD membrane-anchored precursor and is proteolytically processed at the cell surface to yield the mature secreted 17-kD polypeptide. The 80-kD tumor necrosis factor (TNF) receptor ($TNFR_{80}$) is also proteolytically cleaved at the cell surface (shed), releasing a soluble ligand-binding receptor fragment. Since processing of TNF and $TNFR_{80}$ occurs concurrently in activated T cells, we asked whether a common protease may be involved. Here, we present evidence that a recently described inhibitor of TNF processing *N*-{D,L-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl}L-3-(2'naphthyl)-alanyl-L-alanine, 2-aminoethyl amide (TAPI) also blocks shedding of $TNFR_{80}$, suggesting that these processes may be coordinately regulated during T cell activation. In addition, studies of murine fibroblasts transfected with human $TNFR_{80}$, or a cytoplasmic deletion form of $TNFR_{80}$, reveal that inhibition of $TNFR_{80}$ shedding by TAPI is independent of receptor phosphorylation and does not require the receptor cytoplasmic domain.

TNF is a potent mediator of diverse inflammatory and immunomodulatory activities necessary for an effective host defense (1). However, elevated serum levels of TNF seen during many host responses may be harmful or even fatal to the host organism. TNF is initially synthesized as a 26-kD type II transmembrane propeptide which is proteolytically cleaved at the cell surface, releasing the mature 17-kD cytokine assembled as a homotrimer (2). Although both cell surface and secreted forms of TNF appear to be biologically active, it is soluble TNF released into the circulation which may be of primary importance in deleterious physiological responses, such as cachexia or endotoxic shock (3). In support of this concept, a potent and selective metalloprotease inhibitor which blocks cleavage of cell surface TNF in activated macrophages and T cells has been shown to reduce serum TNF levels and increase survival in endotoxin-treated mice (4). More recent evidence indicates that TNF processing is mediated by a Zn^{2+}-dependent endopeptidase related to the matrix metalloproteases (5, 6).

Bioavailability of secreted TNF may be regulated, in part, by naturally occurring soluble TNF-binding proteins generated by proteolytic cleavage (shedding) of cell surface TNF receptors of 55–60 or 75–80 kD ($[TNFR]_{60}$, CD120a; $TNFR_{80}$, CD120b, respectively) (7, 8). Shed TNFR can function as antagonists of TNF biologic activity by saturating receptor binding sites on the ligand competitively inhibiting interaction with cell surface receptors (9). Other evidence suggests that shed TNFR may function as TNF agonists, presumably by stabilizing the TNF trimer at subsaturating concentrations (10). Elevated levels of soluble TNFR have been found in body fluids of patients with elevated TNF levels, suggesting a causal relationship between the two in various pathologic states (11–18). Furthermore, stimuli that induce production of TNF, such as activation of T cells, also trigger proteolytic cleavage of TNFR in vitro, raising the possibility that these processes may be coordinately regulated by a common protease (19).

Previously, we have shown that activated effector T lymphocytes rapidly downregulate $TNFR_{80}$ by shedding before and concurrent with production of TNF (20). Here, we present evidence that a synthetic, hydroxamic acid–based inhibitor of the TNF processing protease, *N*-{D,L-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl}L-3-(2'naphthyl)-alanyl-L-alanine, 2-aminoethyl amide (TAPI) (4), is also a potent antagonist of $TNFR_{80}$ shedding by activated effector T cells. These findings support the concept that these two events are coordinately regulated and possibly mediated by a common cell surface metalloprotease in activated T cells.

Materials and Methods

Reagents and Cells. All cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 μg/ml streptomycin and penicillin and 2 mM glutamine (complete medium).

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PBMC were obtained from healthy volunteers by Ficoll-Hypaque centrifugation (21) and washed and resuspended in complete medium. Effector T cells were obtained by culturing PBMC in complete medium containing anti-CD3 (OKT3) for 3 d followed by complete medium supplemented with rIL-2 (10 ng/ml) for 7 d. These cells were used as a model for differentiated effector T cells and, where indicated, were restimulated with anti-CD3 (20 ng/ml) and PMA (10 ng/ml), as described previously (19). The L929 (murine fibrosarcoma) cell line stably expressing human $TNFR_{80}$ (Lp80) (19) was cultured in complete medium containing 400 μg/ml G418 (GIBCO, Grand Island, NY). Ionomycin was purchased from Sigma Chemical Co. (St. Louis, MO), and PMA was from LC Laboratories (Woburn, MA).

Flow Cytometry. Cells were stained with primary mAbs anti-$TNFR_{80}$ (M1) (20) or anti-TNF (104C) at a concentration of 10 μg/ml for 30 min in ice-cold binding buffer (HBSS supplemented with 10% newborn calf serum, 10 μg/ml human IgG, 20 mM Hepes, pH 7.2, and 0.1% sodium azide). Washed cells were incubated with PE-conjugated goat anti-murine or goat anti-rat IgG (affinity purified, Southern Biotechnology Associates, Birmingham, AL) for 30 min, washed twice, and analyzed directly with a FACScan® instrument (Becton Dickinson, Mountain View, CA). Cell viability was >90% as monitored by propidium iodide staining.

Soluble $TNFR_{80}$ and TNF ELISA. Human peripheral blood T cells (PBT) were isolated via sequential purification with isolymph, SRBC-rosetting, and G10 passage, as previously described (22) PBT were stimulated with OKT3 (10 μg/ml, solid phase) and PMA (10 ng/ml) in the presence or absence of protease inhibitors. Protease inhibitors included TAPI (compound 2 in [4]), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64); *N*-carboxymethyl-Phe-Leu); and α-1-antitrypsin (all from Sigma). Soluble $TNFR_{80}$ and TNF levels were quantitated from culture supernatants obtained 24 h after activation. $TNFR_{80}$ levels were evaluated with a commercial ELISA (R & D Systems, Minneapolis, MN). Human TNF levels were quantitated with antibodies derived at Immunex Corp. (Seattle, WA). Briefly, maxisorp plates (Nunc, Roskilde, Denmark) were incubated overnight with anti-TNF mAb (5 μg/ml). The plates were washed with PBS Tween, and the samples were incubated for 1 h at room temperature. The plates were washed and incubated for 1 h with a 1:500 dilution of rabbit anti-human TNF (P3 ab; Immunex) in PBS Tween containing 5% normal goat serum. The plates were then washed and incubated with a 1:8,000 dilution of horseradish peroxidase–conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Labs., Inc., West Grove, PA) and were developed with tetramethyl benzidine (TMB) substrate system (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD).

Metabolic Labeling and Immunoprecipitation. For pulse-chase analysis, effector T cells (5×10^6/ml) washed in RPMI 1640 and lacking cysteine and methionine were labeled for 30 min with ^{35}S-Cys/Met (160 μCi/ml), then chased in complete medium for various times. Supernatants and detergent extracts of cells were prepared for immunoprecipitation with anti-$TNFR_{80}$ (M1) or anti-TNF (104C) and subjected to SDS-PAGE as described (20). For $^{32}PO_4$ labeling, L929-$TNFR_{80}$ stable transfectants (1.5×10^7/ml) washed in phosphate-free RPMI 1640 were labeled for 60 min with carrier-free $^{32}PO_4$ (0.5 mCi/ml), lysed, and prepared for immunoprecipitation using M1 as described (19).

Radioimmunometric Assay. Surface $TNFR_{80}$ in L929-$TNFR_{80}$ stable transfectants was measured by binding ^{125}I-anti-$TNFR_{80}$ mAb (M1), as described previously (19). Briefly, cells were incubated with 500 pM ^{125}I-M1 for 1 h at 4°C. Nonspecific binding was determined by incubation in the presence of 200-fold molar excess of unlabeled M1. Unbound mAb was removed by four washes with cold binding buffer and bound mAb quantitated by gamma counting.

Results and Discussion

TAPI Inhibits TNF Release and $TNFR_{80}$ Shedding in Activated T Lymphocytes. Resting effector T cells (activated 7 d earlier with anti-CD3) do not secrete detectable levels of TNF and do not express any surface TNF detectable by flow cytometry (Fig. 1 *A*). However, within 2 h of reactivation, using a combination of phorbol ester (PMA) and calcium ionophore, abundant surface TNF was present. Consistent with previous findings (4), surface TNF expression is greatly enhanced when effector T cells are activated in the presence of TAPI, reflecting inhibition of TNF processing.

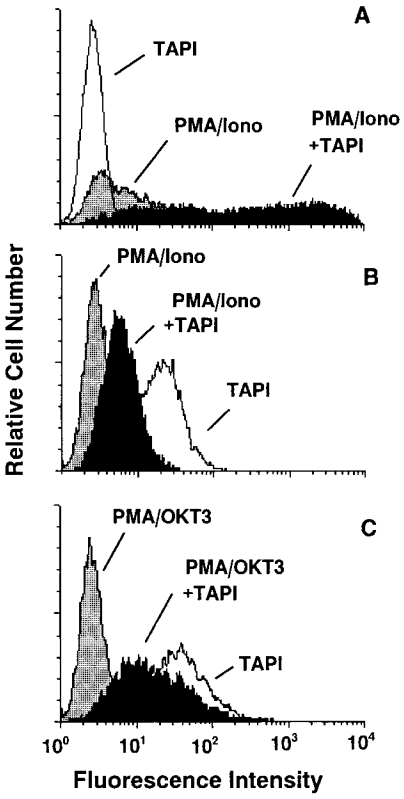
In contrast to TNF, resting effector T cells constitutively express $TNFR_{80}$ on their surfaces. However, reactivation of these cells with PMA/calcium ionophore or PMA/OKT3 triggers rapid downregulation of $TNFR_{80}$ (Fig. 1, *B* and *C* [20]). In the presence of TAPI, activation-induced loss of surface $TNFR_{80}$ was significantly inhibited (Fig. 1, *B* and *C*). A time-course analysis of effector T cells activated with phorbol ester and anti-CD3 (PMA/OKT3) confirmed that TAPI inhibits downregulation of $TNFR_{80}$ (Fig. 1 *D*). However, $TNFR_{80}$ surface expression diminishes even in the presence of TAPI, albeit more slowly and incompletely than cells not treated with TAPI.

Previous studies have established that downregulation of $TNFR_{80}$ in activated effector T cells occurs by shedding, releasing a soluble fragment of the receptor from the cell surface (20). To determine if the inhibition of $TNFR_{80}$ downregulation seen in the presence of TAPI is due to blockade of shedding, soluble $TNFR_{80}$ production was quantitated by ELISA. As shown in Fig. 2 *A*, TAPI caused a dose-dependent reduction in the amount of soluble $TNFR_{80}$ detectable in culture supernatants from activated T cells. In the presence of 200 μM TAPI, shedding of $TNFR_{80}$ was inhibited by ~80%. The concentration of TAPI necessary for half-maximal inhibition of $TNFR_{80}$ shedding is between 25 and 50 μM, which is comparable to the concentration required for half-maximal inhibition of TNF release (~50 μM) by these same cells (4). In addition, examination of various protease inhibitors showed that those which do not block TNF release by activated T cells also fail to prevent $TNFR_{80}$ shedding (Fig. 2 *B*), further supporting the hypothesis that a similar protease mediates both of these processes.

Pulse-chase Analysis of TNF and $TNFR_{80}$ Processing in Activated T Cells. To investigate the effect of TAPI on synthesis and processing of TNF and $TNFR_{80}$ by activated effector T cells in more detail, a pulse-chase labeling experiment was performed (Fig. 3). To study TNF processing, effector T cells activated for 1 h with PMA/OKT3 were pulse-labeled for 30 min with ^{35}S-Cys/Met in the presence or absence of TAPI, then chased with unlabeled Cys/Met. As expected, the 26-kD TNF propeptide was immunoprecipitated only from cell lysates, whereas the 17-kD secreted form of TNF was detected only in the supernatant (Fig. 3 *A*). The rapid loss of 26-kD cell-associated TNF during the chase

period and concomitant accumulation of the 17-kD form in the supernatant, but not intracellularly, indicates that the TNF propeptide is processed to the 17-kD form primarily at the cell surface. In contrast, when the pulse-chase is performed in the presence of TAPI (Fig. 3 *B*), cell-associated-26-kD TNF accumulates, but no secreted 17-kD TNF is detected, demonstrating that TAPI is acting at the cell surface to inhibit TNF processing. Accumulation of the 26-kD cell-associated polypeptide is followed by a gradual decrease in the density of its autoradiographic signal after 20 min, which probably reflects a decrease in specific activity as a consequence of continued protein synthesis during the chase and indicates that TAPI is not inhibiting protein synthesis.

Activated effector T cells rapidly downregulate $TNFR_{80}$ by proteolytic processing of the mature 80-kD protein at the cell surface, releasing a soluble 40-kD fragment of the extracellular domain (Fig. 3 *C*) (20). In the presence of TAPI, however, cell-associated 80-kD TNFR polypeptide accumulates and no soluble 40-kD fragment is detected in the supernatant, even after 80 min, confirming that proteolytic processing of $TNFR_{80}$ is inhibited (Fig. 3 *D*). In spite of this, processing of the 70-kD $TNFR_{80}$ precursor protein to the



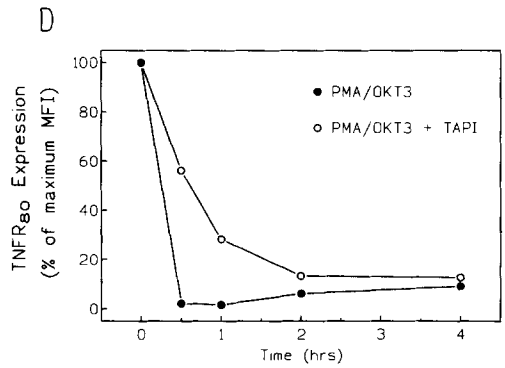


Figure 1. Enhanced expression of surface TNF and inhibition of $TNFR_{80}$ downregulation in activated effector T cells. PBT cells activated with OKT3 and cultured in IL-2 for 7 d were reactivated with a combination of PMA and ionomycin (*A* and *B*) or PMA/OKT3 (*C*) in the presence or absence of TAPI (200 μM). After 2 h, cells were harvested from culture and incubated on ice with either anti-TNF antiserum (*A*), or anti-$TNFR_{80}$ mAb (*B* and *C*) for 30 min, then stained with PE-conjugated goat anti-mouse or anti-rat IgG for 30 min, as described in Materials and Methods. (*D*) PBT cells cultured as described above and reactivated with a combination of PMA and OKT3 in the presence (●) or absence (○) of TAPI (200 μM) were harvested from culture at various times and incubated with anti-$TNFR_{80}$ mAb, then stained with PE-conjugated goat anti-rat IgG for analysis by flow cytometry.

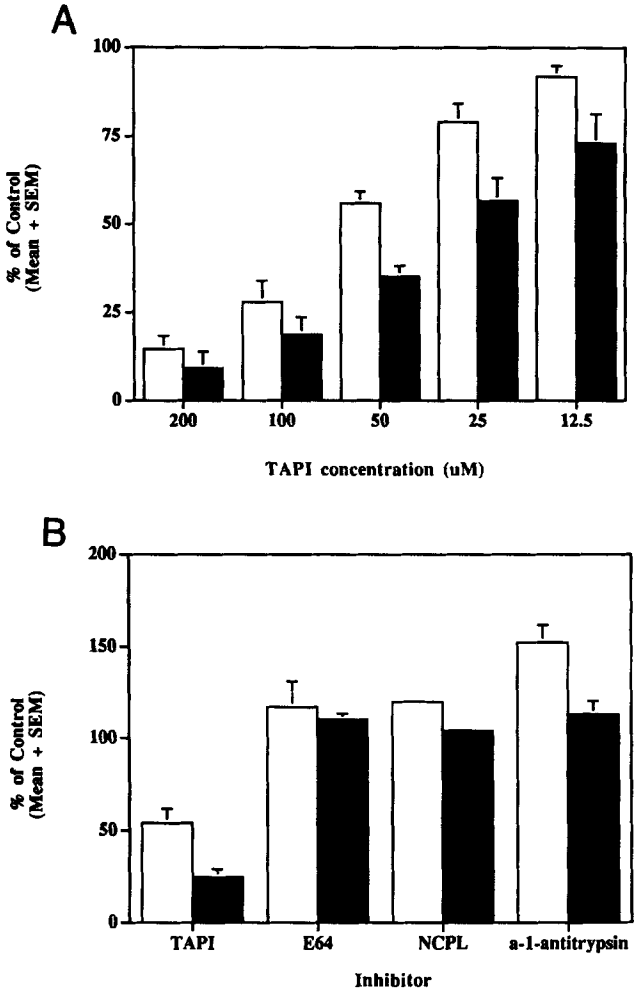


Figure 2. Inhibition of secreted TNF and soluble $TNFR_{80}$ production by TAPI. Human PBTC were stimulated with plate-bound OKT3 and PMA in the presence of (*A*) various concentrations of TAPI or (*B*) other protease inhibitors (50 μM); α-antitrypsin was at 1 μg/ml. Supernatants were harvested and the levels of TNF and soluble $TNFR_{80}$ determined by ELISA. □ TNF, ■ $TNFR_{80}$.

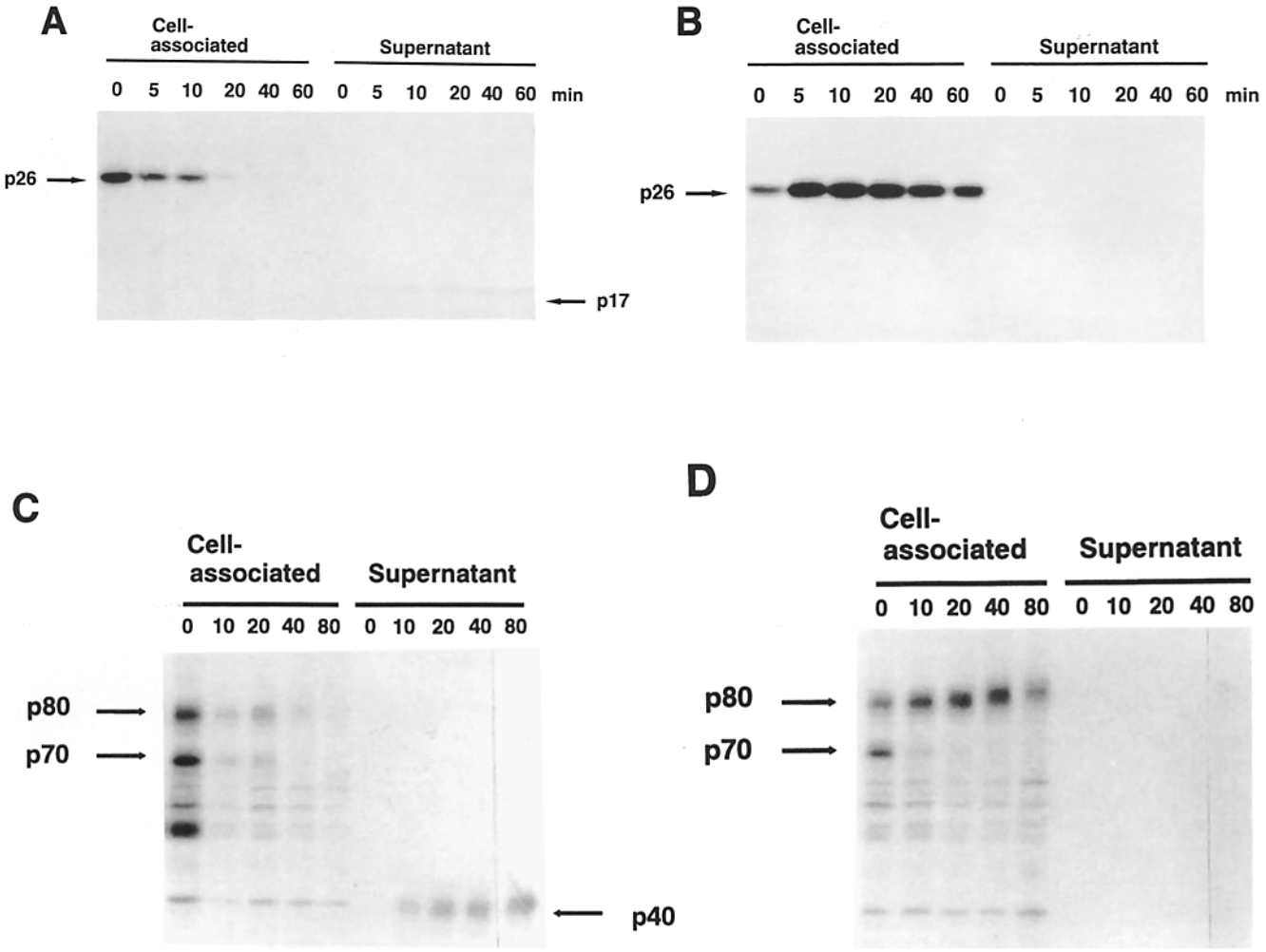


Figure 3. Inhibition of TNF and $TNFR_{80}$ processing in activated T lymphocytes by TAPI. Effector T cells incubated with PMA/anti-CD3 for 60 min were pulse-labeled for 30 min with ^{35}S-Cys/Met in the absence (*A*) or presence (*B*) of TAPI (200 μM), then chased with complete medium ± TAPI. At various times, an aliquot of cells was centrifuged, and TNF immunoprecipitated from the cell-free supernatants or from cell lysates, using rabbit anti-TNF antiserum, was resolved by SDS-PAGE (12% gel) and autoradiography (8-d exposure). Processed TNF lacks two met residues, accounting for the lower autoradiographic band density relative to the 26-kD form. Effector T cells were pulse-labeled for 30 min with ^{35}S-Cys/Met in the absence (*C*) or presence (*D*) of TAPI (200 μM), then chased with complete medium containing PMA/anti-CD3 ± TAPI. At various times, an aliquot of cells was centrifuged to prepare cell lysates and cell-free supernatants for immunoprecipitation, using anti-$TNFR_{80}$ mAb (M1). $TNFR_{80}$ was resolved by SDS-PAGE (10% gel) and autoradiography of the dried gel (10-d exposure).

80-kD form continues in the presence of TAPI, demonstrating that maturation of $TNFR_{80}$ is not inhibited. Collectively, these findings clearly show that TAPI inhibits shedding of $TNFR_{80}$ and suggest that the initial inhibition of downregulation seen by flow cytometric analysis is also due to inhibition of shedding. The incongruity between surface staining and measurement of soluble receptor levels at later time points indicates that, when shedding is blocked, $TNFR_{80}$ may be downregulated by an alternate process. In this regard, mutations in $TNFR_{60}$, which inhibit proteolytic cleavage, do not block downregulation in response to PMA, indicating that multiple mechanisms for downregulating TNFR are operative (23). Furthermore, we have observed that TAPI has no effect on expression of CD4 which is downregulated by internalization after T cell activation (24), but TAPI partially inhibits downregulation of $TNFR_{60}$, which is also subject to proteolytic cleavage (25) (data not shown) on neutrophils.

TAPI Inhibits Shedding of $TNFR_{80}$ in L929-$TNFR_{80}$-stable Transfectants. To further investigate the mechanism of inhibition of $TNFR_{80}$ shedding in a more defined cellular system, mouse L929 cells stably transfected with human $TNFR_{80}$ (Lp80) were activated with phorbol ester (PMA, 100 ng/ml) for 30 min in the presence or absence of TAPI, and surface $TNFR_{80}$ levels were detected by a radioimmunometric binding assay, using anti-$TNFR_{80}$ mAb. As shown in Table 1, Lp80 shed ~73% of their receptors after stimulation with PMA, whereas, in the presence of TAPI, they shed only ~27% of their $TNFR_{80}$ (63% inhibition). Thus, the protease which regulates shedding of human $TNFR_{80}$ expressed in mouse cells is also sensitive to inhibition by TAPI.

Table 1. *Shedding of $TNFR_{80}$ by Lp80 and Lp80Δcyt*

		$TNFR_{80}$ expression (^{125}I-M1 bound, cpm)	
		Control	TAPI
Lp80	−PMA	21,909 ± 212	20,466 ± 297
	+PMA	5,872 ± 206 (73)	14,973 ± 417 (27)
Lp80Δcyt	−PMA	20,807 ± 1,012	21,572 ± 861
	+PMA	12,415 ± 624 (40)	17,762 ± 118 (18)

Cells (1.5 × 10^5/well in 24-well plates) were preincubated with media only (control), and, then, TAPI (20 μM) for 90 min at 37°C, before activation with PMA (100 ng/ml) for 30 min. Specific ^{125}I-M1 binding was determined by incubating cells with ^{125}I-M1 (500 pM) for 1 h at 4°C. The results shown are means ± SEM of two separate experiments. Nonspecific binding cpm determined in the presence of 200-fold excess unlabeled M1 were 1,743 ± 106 for Lp80, and 2,289 ± 52 for Lp80Δcyt. Numbers in parentheses represent percent loss of specific ^{125}I-M1 binding compared to unactivated cells.

Previously, we have shown that $TNFR_{80}$ is constitutively phosphorylated and that phosphorylation is a late processing event which precedes proteolytic cleavage of $TNFR_{80}$ (19). In addition, constitutive phosphorylation and shedding of $TNFR_{80}$ were blocked by the protein kinase inhibitor staurosporine, suggesting a link between receptor phosphorylation and proteolysis. As observed in Fig. 3 *D*, the gradual increase in apparent molecular mass of T cell–associated $TNFR_{80}$ in the presence of TAPI suggested that phosphorylation of $TNFR_{80}$ continues, even though shedding is blocked. However, to directly examine whether TAPI affects phosphorylation of $TNFR_{80}$, Lp80 cells were labeled with [^{32}P]-orthophosphate in the presence or absence of TAPI before treatment with PMA. Cell extracts were then subjected to immunoprecipitation using M1. As shown in Fig. 4, TAPI did not inhibit constitutive phosphorylation of $TNFR_{80}$, but did block activation-induced loss of phosphorylated receptor. To confirm that inhibition of $TNFR_{80}$ shedding by TAPI is independent of receptor phosphorylation, L929 cells expressing a mutant form of the receptor lacking the entire cytoplasmic domain (Lp80Δcyt) were tested for activation-induced loss of $TNFR_{80}$ in the presence or absence of TAPI. Truncated

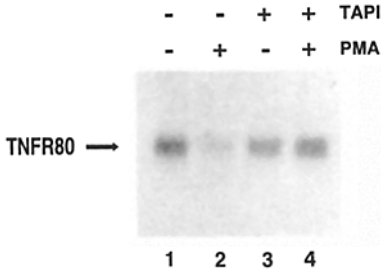


Figure 4. L929-$TNFR_{80}$ stable transfectants were washed in phosphate-free medium, labeled with [^{32}P]-orthophosphate for 2 h, then incubated without (lanes *1* and *2*) or with (lanes *3* and *4*) TAPI for 5 min before addition of PMA (100 ng/ml) (lanes *2* and *4*), or nothing (lanes *1* and *3*). After 30 min further incubation, detergent extracts of cells were subjected to immunoprecipitation with anti-$TNFR_{80}$ mAb followed by SDS-PAGE (8% gel) and autoradiography (5-h exposure).

$TNFR_{80}$ expressed by these cells is not phosphorylated but is shed in response to PMA, albeit more slowly and incompletely compared to cells expressing the full-length receptor (19). As seen in Table 1, Lp80Δcyt shed ~40% of their $TNFR_{80}$, but, in the presence of TAPI only, ~18% was shed (55% inhibition), clearly demonstrating that inhibition of $TNFR_{80}$ shedding by TAPI does not involve the receptor cytoplasmic domain, and, therefore, is independent of receptor phosphorylation.

In conclusion, the data presented here show that a cell-surface metalloprotease is involved in shedding of $TNFR_{80}$ by activated T lymphocytes. Furthermore, the observation that TNF processing and $TNFR_{80}$ shedding occur concurrently and are similarly sensitive to inhibition by TAPI strongly suggests that these processes are coordinately regulated during T cell activation. Notably, the protease cleavage site in TNF (Ala-Val) is also present in the extracellular domain of $TNFR_{80}$ (Ala_{213}-Val_{214}) at a site consistent with the observed molecular weight of the shed receptor fragment (26). Thus, metalloprotease inhibitors such as TAPI may offer protection from the deleterious systemic effects of TNF at two levels simultaneously: first, by preventing the release of soluble TNF, and second, by blocking accumulation of shed TNFR; soluble TNFR has been shown to stabilize TNF activity in vitro (10). It will be of interest to determine whether other members of the TNF ligand family or the TNFR family are also subject to coordinated proteolytic processing.

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