# Further evidence for a common mechanism for shedding of cell surface proteins

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**Abstract Pro-TNFα, Steel factor, type II IL-1R and IL-2Rα were expressed in COS-7 cells and the generation of their soluble forms was examined. The release of all four proteins was strongly stimulated by the phorbol ester PMA and completely blocked by a hydroxamate-based inhibitor of metalloproteases. COS-7 cell membranes were found to cleave various synthetic pro-TNFα peptides with the same specificity as a partially purified TNFα converting enzyme purified from human monocytic cells, suggesting that the same enzyme may be responsible for at least some of the COS-7 cell shedding activity.**

*Key words:* Limited proteolysis; Metalloprotease; Protease inhibitor; Shedding

## 1. Introduction

Soluble forms of many cytokine receptors and growth factors with membrane-bound precursors are generated by limited proteolysis of the membrane-associated protein; this process has been called shedding (for reviews see [1,2]). The structural requirements for shedding appear to vary. No consensus amino acid sequence has been found at the proteolytic cleavage sites of cytokine receptor and growth factor precursors identified so far [1,2]. It was demonstrated that the presence or absence of the cytoplasmic domains of the IL-6R [3] and the p60 TNFR [4] had no effect on shedding of these two proteins, whereas in the case of the p80 TNFR [5] the intracellular part of the molecule influenced the generation of the soluble receptor. Furthermore it has been found that the C-terminal valine residue of the cytoplasmic tail of TGFα is critical for the release of this growth factor [6].

In contrast to the differences with respect to the structural requirements for shedding, the release of most soluble cytokine receptors and growth factors is strongly enhanced by PMA (for reviews see [1,2]), a potent activator of protein kinase C [7,8], indicating that the underlying mechanisms are similar. Another line of evidence suggesting that the proteolytic activities responsible for various shedding events are closely related comes from experiments with a hydroxamate-based metalloprotease inhibitor we named TAPI (for 'TNFα protease inhibitor'). This compound prevents the release of TNFα from a monocytic cell line and protects mice against a lethal dose of endotoxin [9–11]. Interestingly, we found that TAPI also blocks shedding of the p80 TNFR from human T-cells [12] and shedding of the p60 TNFR and IL-6R from transfected cells and human primary monocytes [13]. A recent study demonstrated that TAPI blocks the release of L-selectin and TGFα from transfected cells [14] and hydroxamates have also been shown to inhibit L-selectin shedding from primary cells [15–17]. Other proteins whose release is blocked by TAPI or related compounds include the thyrotropin receptor [18], Fas ligand [19], heparin-binding EGF-like growth factor [20] and angiotensin-converting enzyme [21]. No inhibitors other than hydroxamates have been found to prevent these shedding events under physiological conditions.

Here we show that TAPI also inhibits shedding of Steel factor, type II IL-1R and IL-2Rα from transfected COS-7 cells. In addition we demonstrate that the simian kidney cell line COS-7 and the human monocytic cell line THP1 possess metalloprotease activities with identical substrate specificity for pro-TNFα peptides.

## 2. Materials and methods

### 2.1. Reagents

The metalloprotease inhibitor TAPI was prepared at Immunex Corporation [9]. PMSF, leupeptin and pepstatin were obtained from Boehringer Mannheim (Indianapolis, IN). PMA was from Sigma (St. Louis, MO). [$^{35}$S]Cysteine (> 1000 Ci/mmol) and [$^{35}$S]methionine (> 1000 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). The generation of expression vectors for murine Steel factor [22], the human type II IL-1R [23] and the human IL-2Rα [24] was described previously. The cDNA containing the entire coding regions of the membrane-bound form of human TNFα was cloned into the mammalian expression plasmid pDC303 [25]. The preparation of a monoclonal antibody directed against the type II IL-1R was previously described [26]. A rabbit antiserum specific for murine Steel factor was raised using murine recombinant Steel factor. A TNFR-Fc fusion protein was obtained as described [27].

### 2.2. Culture and transient transfection of COS-7 cells

COS-7 cells were grown in DMEM/F12 (1:1) (Gibco-BRL, Grand Island, NY) containing 5% FCS (Intergen, Purchase, NJ), 50 U/ml penicillin and 50 μg/ml streptomycin at 5% $CO_2$ in a water saturated atmosphere. Transient transfections of COS-7 cells using DEAE/dextran were performed as described [23]. After transfection cells were cultured for 48 h. The cells were metabolically labeled and stimulated under the conditions indicated in the figure legends.

### 2.3. Immunoprecipitation of proteins

COS-7 cells were metabolically labeled with [$^{35}$S]cysteine/methionine under the conditions indicated in the legend to Fig. 1. Culture supernatants were harvested and the cells were lysed in RIPA buffer (PBS containing 1% NP40, 1% 7-deoxycholic acid, 0.1% SDS) supplemented with protease inhibitors (EDTA, 10 mM; leupeptin, 0.5 μg/ml; pepstatin, 0.7 μg/ml; PMSF, 35 μg/ml). To the culture media NP40 was added to a final concentration of 1%. Cell lysates and media were incubated with appropriate antibodies for 2 h at 4°C. In order to detect TNFα a TNFR-Fc fusion protein was used. Immunocomplexes were precipitated with protein A-Sepharose (Pharma-

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*Abbreviations:* TNFR-Fc, soluble form of the p80 TNFR linked to the constant region of the human Ig heavy chain


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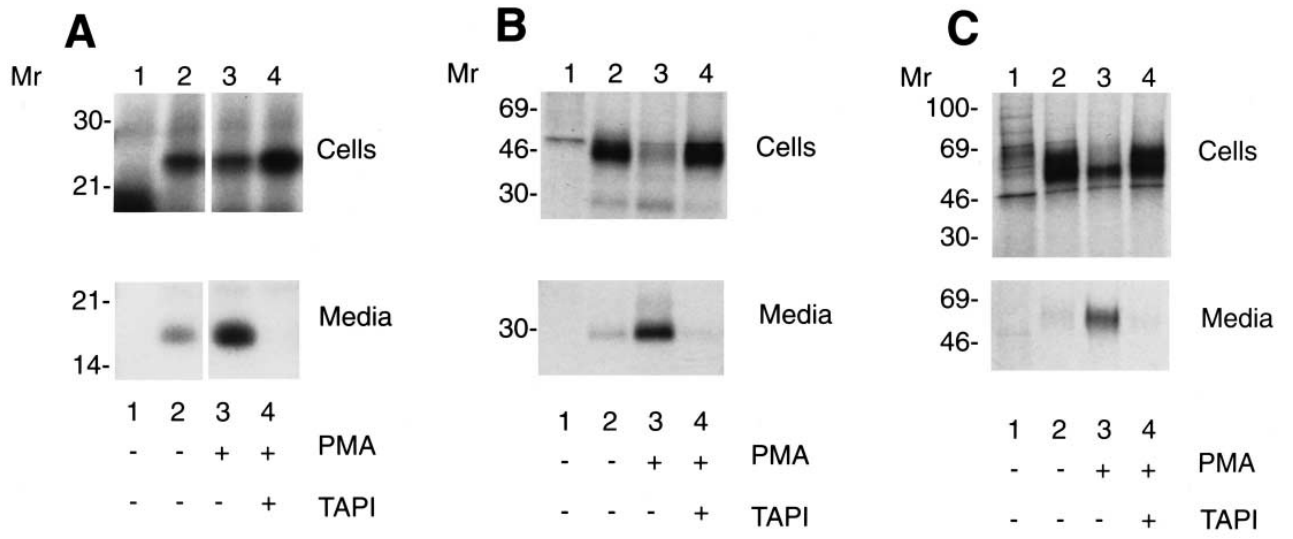


Fig. 1. Inhibition of shedding of TNFα (A), Steel factor (B) and type II IL-1R (C) in transfected COS-7 cells by TAPI. COS-7 cells ($2\times10^6$ cells) transiently transfected with cDNAs coding the membrane-bound forms of TNFα, Steel factor or type II IL-1R were metabolically labeled with 50 μCi of a mixture (1:1) of [$^{35}$S]cysteine and [$^{35}$S]methionine for 2 h in methionine/cysteine-free media. Cells were then incubated in media containing unlabeled methionine/cysteine for 1 h in the presence and the absence of PMA (10 ng/ml) and TAPI (200 μM) as indicated. TNFα, Steel factor and type II IL-1R proteins were immunoprecipitated from cell lysates and media using TNFR-Fc fusion protein (A) and antibodies specific for Steel factor (B) or type II IL-1R (C) and analyzed by SDS-PAGE and fluorography. Untransfected COS-7 cells were used as a control (lanes 1).

cia, Piscataway, NJ) and separated on 8–16% SDS polyacrylamide gels (Novex, San Diego, CA) [28]. After treatment with fluorographic intensifier solution 'Amplify' (Amersham) gels were dried and exposed to X-ray film.

*2.4. ELISA for soluble IL-2Rα*

The levels of soluble IL-2Rα in culture media were determined using an ELISA kit purchased from Boehringer Mannheim following the manufacturer's instructions.

*2.5. Analysis of TNFα peptide digests*

Peptides were synthesized and digests were analyzed, by reverse phase HPLC, as previously described [29]. Digests were carried out in a volume of 20 μl, containing 0.5 mM peptide, 10 mM Tris-HCl (pH 8), 1% octylglucoside, for 4 h at 37°C. COS-7 cell membranes were prepared as previously described [9] and concentrated to 20 μg/ml. TNFα converting enzyme (TACE) was prepared as previously described [9].

## 3. Results and discussion

We assayed the release of TNFα, Steel factor, type II IL-1R and IL-2Rα from COS-7 cells transiently expressing the transmembrane forms of these proteins. As we reported previously [13], TNFα is shed from the transfected cells spontaneously and its release is strongly enhanced by PMA (Fig. 1A, lanes 2, 3). The metalloprotease inhibitor TAPI prevents spontaneous shedding of TNFα (data not shown) as well as the PMA-induced release of TNFα (Fig. 1A, lane 4).

In the experiments shown in Fig. 1B,C, we investigated the influence of TAPI on shedding of Steel factor (Fig. 1B) and type II IL-1R (Fig. 1C). In cell lysates, Steel factor is detected as a 45 kDa protein and the type II IL-1R as a 67 kDa protein; the corresponding soluble proteins migrate at apparent molecular weights of 31 kDa and 55 kDa. In contrast to the release of TNFα, spontaneous shedding of both proteins occurs at a low level (lane 2). Shedding of Steel factor and type II IL-1R is strongly enhanced by PMA (lane 3), and this induced release is completely blocked by TAPI (lane 4). In the cell lysates of COS-7 cells expressing the type II IL-1R, a second immunoreactive protein with higher electrophoretic mobility was detected (Fig. 1C). This protein band may represent a biosynthetic precursor of the type II IL-1R which is not expressed at the cell surface, since its level is unaltered when cells are treated with PMA and TAPI.

As shown in Fig. 2, TAPI also strongly inhibits the spontaneous and PMA-induced release of soluble IL-2Rα from COS-7 cells transfected with a cDNA coding the membrane-bound protein. The level of soluble IL-2Rα detected in media of the transfected cells when cultured in the presence of TAPI was always about 20–25% of the amount in the untreated control.

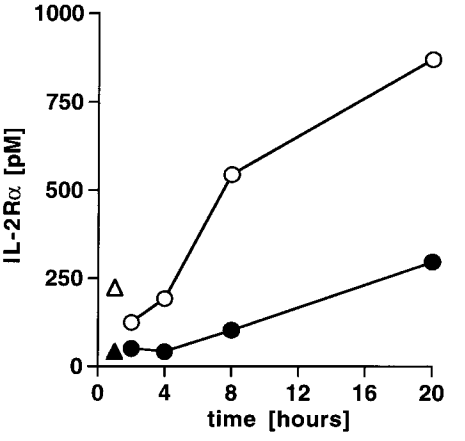


Fig. 2. Inhibition of shedding of IL-2Rα in transfected COS-7 cells by TAPI. COS-7 cells ($2\times10^6$ cells) transfected with the cDNA coding the IL-2Rα were either treated for 1 h with PMA (10 ng/ml) in the absence (open triangle) or presence (filled triangle) of TAPI (200 μM) or were cultured without PMA in absence (open circles) or presence (filled circles) of TAPI (200 μM) for the times indicated. The concentrations of soluble IL-2Rα in the culture media were determined using a specific IL-2Rα ELISA.

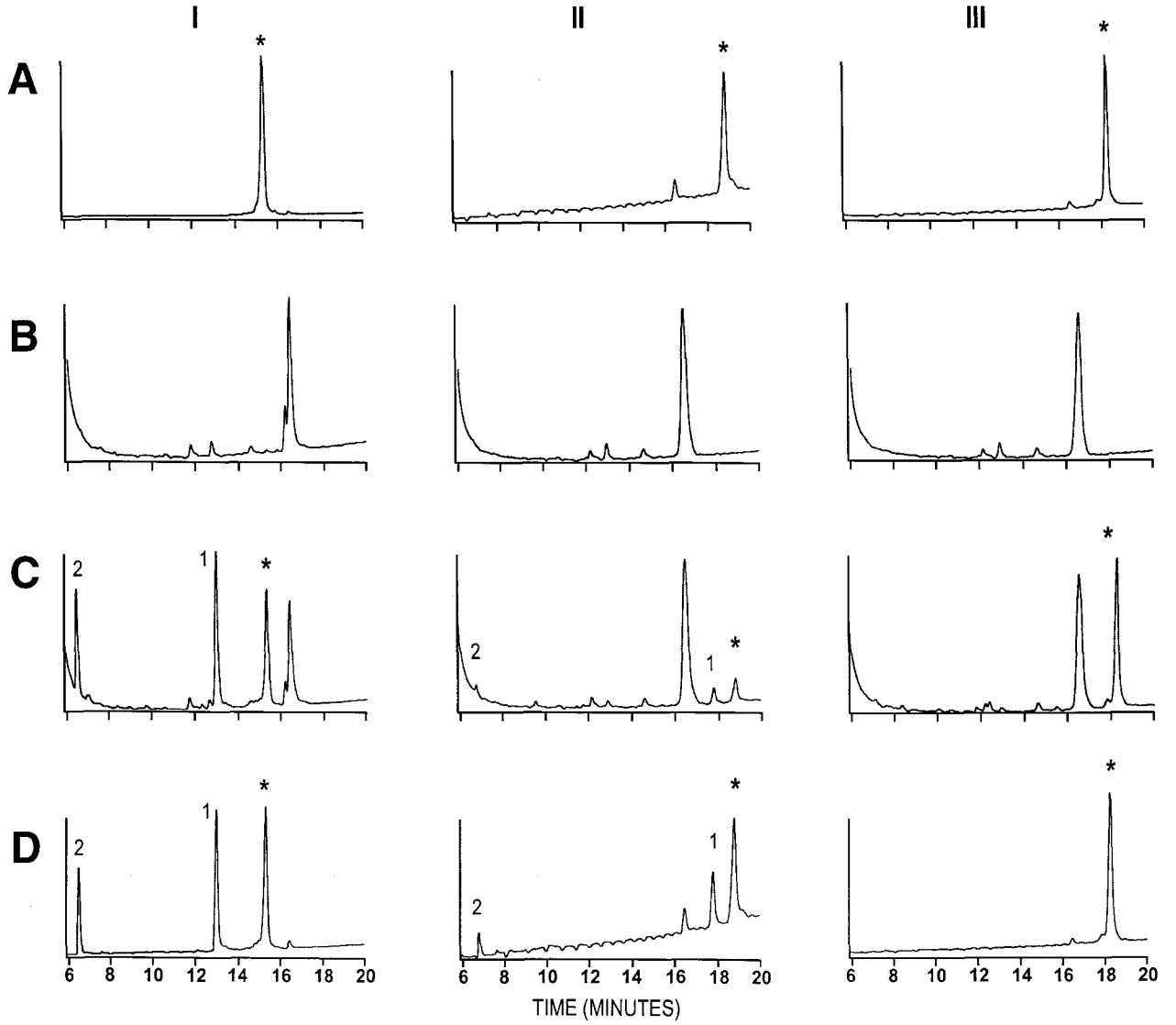


Fig. 3. HPLC analysis of digests of TNFα peptides. Columns: I, Ac-SPLAQAVRSSSR-$NH_2$; II, Ac-SPLAQFVRSSSR-$NH_2$; III, Ac-SPLAQIVRSSSR-$NH_2$. Rows: A, peptide incubated with buffer; B, COS-7 cell membranes incubated with buffer; C, peptide incubated with COS-7 cell membranes; D, peptide incubated with TACE. The asterisks indicate the peptide substrates and the numbers indicate the products generated. The vertical axis represents relative absorbance, rather than specific units, because the absorbance of the three peptide substrates varied widely.

To determine whether at least one of the shedding enzymes in COS-7 cells could be the TACE that we have partially purified from THP-1 cells [9], we compared the ability of COS-7 membranes and TACE to cleave a number of pro-TNFα peptides. Both preparations generated the same products from a peptide representing the cleavage site in pro-TNFα (Fig. 3, column I), and both also generated the same products from an altered peptide in which Phe replaces the alanine N-terminal to the cleaved peptide bond (P1 Ala residue) (Fig. 3, column II). In all cases the observed peptide cleavage was sensitive to inhibition by TAPI (data not shown). Finally, both preparations failed to cleave a peptide in which Ile replaces the P1 Ala residue (Fig. 3, column III).

We have shown previously that the release of TNFα, TNFRs and IL-6R from transfected cells and human primary cells is inhibited by TAPI [12,13]. In this study we showed that TAPI also blocks the release of Steel factor, type II IL-1R and IL-2Rα from transfected COS-7 cells. The finding that the release of the soluble forms of all these molecules can be strongly accelerated by PMA ([5,13]; this study) and can be blocked by TAPI suggests that a common mechanism is involved, possibly one (or a few) metalloprotease(s) with a broad substrate specificity. This view is also supported by results obtained with CHO cells and a mutant cell line deficient in shedding of TGFα and β-amyloid precursor protein [30]. The investigators found that many endogenous surface proteins on CHO cells are shed in response to PMA and that the mutant cell line failed to release any of these proteins. Alternatively, the existence of a family of metalloproteases, each responsible for shedding of a distinct membrane protein and sensitive to TAPI but not other metalloprotease inhibitors, cannot be excluded.

Our results suggest that the same enzyme that releases TNFα is present in both a simian kidney cell line and human monocytic cells. For TNFα, at least, there may be a single shedding mechanism. The biological function of shedding still

remains unclear. Many molecules which are generated by limited proteolysis such as soluble cytokine receptors and growth factors play important roles in certain biological systems [1,2]. On the other hand, the same stimuli may lead to the release of several surface proteins with various or even opposite functions, e.g. the release of both TNFα and the two TNFRs (which might be able to neutralize TNF activity) from primary human cells can be stimulated by PMA. Since some spontaneous shedding activity is observed in cell lines such as COS-7 and THP1 cells, it is possible that shedding might be important for the transformation of cells. Shedding of cell surface molecules might allow tumor cells to escape detection by the immune system.

Eventually the molecular cloning of individual shedding proteases and characterization of their substrate specificity will lead to a better understanding of their functions and also allow the rational design of specific inhibitors.

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