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ARTICLES

# UVC Activation of the HeLa Cell Membrane "TGFαASE," a Metalloenzyme

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**Abstract** We have investigated the effect of UVC irradiation on "TGFαase" activity using both intact HeLa cells and isolated membrane fragments with an assay based on the previously described nonapeptide substrate method [Brown et al. (1992): J Cell Biochem 48:411–423]. This method allows recognition of cleavage at the scissile bond cognate with that of the TGFα N-terminal cleavage site from its membrane-bound precursor. The level of ectoendopeptidase (including "TGFαase") activity observed on intact cells was lower than that of ectoaminopeptidases. Addition of foetal bovine serum (FBS) enhanced aminopeptidase and dipeptidyl peptidase activity but inhibited "TGFαase" activity, while phorbol 12-myristate 13-acetate (PMA) had no significant effect on the ectopeptidases monitored, except for "TGFαase," which was also inhibited, in contradistinction to their effects in other cell systems. Sublethal UVC irradiation (10 $Jm^{-2}$) of the cultures resulted in activation of the ectoaminopeptidase and ectoendopeptidases which was maximal 16 and 20–24 h after irradiation, respectively. The addition of FBS to these irradiated cells appeared to reduce the increase in endopeptidase products, due in part to increased aminopeptidase activity but also to the direct inhibitory effect of FBS on the "TGFαase." The activation of these proteases by UVC, even at high fluences (500 $Jm^{-2}$), was not observed within the first 30 min after the cells were irradiated. Purified plasma membrane fragments were prepared from suspension cultures of HeLa cells and displayed high levels of "TGFαase" activity. The rate of "TGFαase" activity using 140 nM peptide substrate (P9) was 5.6 pmol/min/mg membrane protein, which was elevated to 13.7 pmol/min/mg membrane protein, 20 h after the cells had been irradiated with 10 $Jm^{-2}$ UVC. Inhibition studies indicate that the plasma membrane "TGFαase" is a metalloenzyme, as it was inhibited by EDTA, EGTA, and 1,10-phenanthroline but not by elastase or serine protease inhibitors. "TGFαase" activity on intact cells was shown to be inhibited by 1,10-phenanthroline, which further supports this suggestion. Treatment of the membranes with Triton X-100 resulted in a loss of "TGFαase" activity, raising the possibility that this enzyme may require a cofactor to be fully functional. We show that in purified membrane preparations of HeLa cells there is evidence for the presence of a "TGFαase" which can be activated by UV irradiation, which differs from the putative "TGFαase" described in various other cell lines, and which does not seem dependent on protein kinase C (PKC) activity. J. Cell. Biochem. 64:353–368. 

**Key words:** transforming growth factor α; "TGFαase"; ultraviolet radiation; cell surface proteases; HeLa cells; membrane fragments

Transforming growth factor α (TGFα) is a member of a class of growth factors which is synthesised as a membrane-anchored precursor consisting of an extracellular growth factor domain, a single transmembrane domain, and a cytoplasmic domain [Massagué, 1990; Derynck, 1992; Massagué and Pandiella, 1993; Lee et al., 1995]. PreproTGFα is synthesised intracellularly and transported to the cell surface as a glycosylated transmembrane protein of 18–22 kDa, which is rapidly converted in some cell lines to a nonglycosylated 17 kDa transmembrane protein by cleavage of the N-terminal side of the TGFα moiety upon reaching the cell surface [Teixidó et al., 1990; Pandiella and Massagué, 1991a,b; Arribas and

Abbreviations used: BCA, bicinchoninic acid; CHO cells, Chinese hamster ovary cells; EGF, epidermal growth factor; FBS, foetal bovine serum; HBSS, Hank's balanced salt solution; PBS, phosphate buffered saline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulphonyl fluoride; TGFα, transforming growth factor α.

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Massagué, 1995]. The 17 kDa form is in turn converted into the soluble mature 6 kDa form of TGFα, the control of which cleavage is regulated by the C-terminal valine residue of prepro-TGFα [Bosenberg et al., 1992]. The N-terminal cleavage site is between the alanine-valine link at positions 38 and 39, while the C-terminal cleavage site is between alanine-valine at positions 88 and 89 of the preproTGFα moiety [Derynck et al., 1984; Wong et al., 1989]. The rate of cleavage at the C-terminal site is slow under basal conditions but is increased by agents such as phorbol ester, calcium ionophore, and serum in Chinese hamster ovary (CHO) cell cultures and some cultured derivatives of breast and colon carcinomas [Pandiella and Massagué, 1991a,b; Pandiella et al., 1992; Harano and Mizuno, 1994; Levine, 1994; Ramesh and Levine, 1995].

The cell surface protease(s) responsible for processing TGFα from its precursor prepro-TGFα remains to be identified. It has been shown that an elastase-like enzyme in CHO cells (used as a model system but not normally expressing TGFα [Teixidó et al., 1990]) cleaves overexpressed, transduced TGFα from its precursor molecule [Pandiella et al., 1992; Harano and Mizuno, 1994; Cappelluti et al., 1993]. In other studies, the release of 7-amino-4-methylcoumarin from the fluorescent substrate Suc-Ala-Ala-Ala-MCA has been used to measured enzyme activity [Harano and Mizuno, 1994; Levine, 1994]. It must be emphasized that there is significant variation in the source of cultured cells used to analyse TGFα processing and that, while human breast tumour epithelium is normally stimulated by EGF/TGFα and the expressed EGF receptors are modulated by hormonal levels [Salmonon et al., 1984; Derynck et al., 1987], the most frequently used cell line—CHO—is not [Teixidó et al., 1990].

Sublethal doses of UVC resulted in an increased TGFα release from HeLa cells, melanocyte, and melanoma cell lines [Ellem et al., 1988], which may well be the trigger for the known epidermal mitogenesis seen following UV irradiation of the skin [Pearse et al., 1987; Stierner et al., 1989]. Such epidermal cell multiplication is concerned with the immediate tissue repair function of replacement of dying "sunburn cells" [Danno and Horio, 1987]. The immediate need for cell replacement, requiring rapid cell cycling, may carry long-term penalties as a consequence of TGFα-induced DNA synthesis before all UV-induced DNA lesions have been repaired. Increased mutation rates in the siblings from such cells would confer on them a higher probability of developing preneoplastic clones and thus contribute to the later development of sunlight-associated skin cancers. Repeated episodes of sunburn lead to TGFα release in vivo [Ellem et al., unpublished] and could add further penalty by promoting clonal evolution to malignancy by the episodes of TGFα-driven epidermal cytokinesis. Soluble TGFα is 100-fold more effective as a mitogen than its membrane-bound precursor [Brachmann et al., 1989], so the release of TGFα from its precursor may be a major control point for its mitogenic activity. This increase in TGFα release from melanoma cells was due to a post-translational mechanism that was not reliant on de novo protein synthesis [Chenevix-Trench et al., 1992]. Brown et al. [1992, 1993] showed that not only did the activity of the cell surface endopeptidases which may be responsible for cleaving the TGFα analogue but also ectoaminopeptidase activity increased severalfold after UVC exposure.

In this study we have separated the membranes from tissue-cultured cells which have been exposed to a variety of agents, including UVC irradiation and phorbol esters. "TGFαase" activity in these fragments was assayed using the nonapeptide method. We found a high level of "TGFαase" activity in HeLa cell membranes which increased after the cells were exposed to UVC. This enzyme has the characteristics of a metalloenzyme, not a serine protease.

## MATERIALS AND METHODS

### Materials

All the chemicals and biochemicals were obtained from Sigma (St Louis, MO), except for the following: trypsin, penicillin, and streptomycin which were obtained from Commonwealth Serum Laboratories (Melbourne, Australia), RPMI tissue culture medium obtained from Gibco (Grand Island, NY), foetal bovine serum (FBS) obtained and processed inhouse, Whatman channelled TLC plates (LK6D or LK6DF) obtained from FSE Scientific (Brisbane, Australia), $Na^{125}I$ (100 mCi/ml) and [U-$^{14}$C]5′-AMP (20 μCi/ml) obtained from NEN/DuPont (Boston, MA), and chloramine-T obtained from BDH Chemicals Ltd (Poole, England). All other reagents were of AR grade. All aqueous solutions were prepared with Milli-Q purified water.

### Peptide Iododination

The nonapeptide $H_2N$-YVAAAVVSH-$NH_2$ (denoted P9 herein) was prepared and purified within this institute prior to being iodinated using $Na^{125}I$ as previously described [Brown et al., 1992].

### HeLa Cell Culture

HeLa $S_3$ cell (ATCC CCL 2.2) monolayers were grown in RPMI 1640 containing penicillin (100 µg/ml), streptomycin (100 µg/ml), and 5% (v/v) FBS (pH 7.2 at 37°C) under sterile conditions. HeLa cells were also grown in 1 l suspension culture flasks (Bellco, Vineland, NJ) using the same medium described above. Every day approximately 20% of the suspension was decanted from the flask and replaced by an equal volume of fresh medium. The cells were harvested twice weekly when 700–800 ml of the cell suspension was removed from the flask and replaced by an equal volume of fresh medium. Cell viability was routinely monitored to ensure maximal viability was being maintained in culture.

### Peptidase Activity

**Cell monolayers.** HeLa cells grown to near confluency in 24-well plates were placed in either a 37°C incubator or on ice for 30 min to allow for temperature equilibration to occur. At time zero, the medium in the wells was aspirated, and the cells washed with phenol red–free HBSS (hereafter called HBSS) (at the relevant temperature) to remove residual traces of RPMI 1640. The cells were then incubated for several intervals up to 60 min at 37°C or 4°C in 200 µl HBSS containing 10 pmoles $^{125}$I-labelled P9 (final concentration 50 nM). At the end of the incubation time, the medium was collected and stored on ice, and 40 µl was applied to a TLC plate. Label present in a 40 µl sample was measured using a γ-counter. The cells were then washed three times with ice-cold HBSS and the protein solubilised by the addition of 0.5 ml 0.1 M NaOH. Aliquots of the cell digest were spotted (40 µl) onto TLC plates, assayed for protein content (10 µl), and measured for incorporation of $^{125}$I label with the γ-counter (250 µl). HeLa cell protein was measured using the bicinchoninic acid (BCA) assay (Sigma) with BSA as the standard. The hydrolysis of P9 by the various ectopeptidases was linear up to 70% P9 hydrolysis [Brown et al., 1992].

The effect of FBS (0–10% v/v), PMA (1 µM), and/or bestatin (10 µM) on peptidase activity was determined by adding these compounds to the labelled medium just prior to addition to 4 h FBS-depleted cell cultures.

**Suspension cells and membrane fragments.** Aliquots from each step of the membrane isolation procedure were assayed for their ability to cleave P9. In this assay, 20 µl of labelled P9 (1 µl $^{125}$I-P9 (10 pmoles) in 20 µl HBSS) was added to a 1.5 ml Eppendorf tube along with 50 µl of the membrane fraction (prewarmed to 37°C for 15 min) for 30 min (P9 final concentration of 140 nM). At the end of the experiment 40 µl of the reaction mixture was spotted on a multichanneled TLC plate. Bestatin was added to the assay to a final concentration of 10 µM. A 10 µl aliquot was removed from the Eppendorf tubes for γ-counting.

It was noted that there was a 3–4 min delay between spotting the membrane sample and the plate drying, which was shown to allow continuation of P9 hydrolysis which affected the determination of the rate of P5 hydrolysis of these fractions. In order to correct for this, membrane fractions and $^{125}$I-P9 were simultaneously spotted onto the TLC plate and allowed to dry. During this period 28.0 ± 0.9 (n = 6, SEM) and 61.0 ± 3.1 (n = 6, SEM) pmole P5/mg membrane protein were formed for the sham- and UVC-irradiated cultures, respectively. These values were used to correct for the rate of P5 formation during time course assays of P9 hydrolysis by membrane fragments.

### TLC Peptide Analysis

Medium and cellular extracts were applied to channelled TLC plates and separated by ascending chromatography (butanol:$H_2O$:acetic acid, 100:30:10 (BAW), pH 2.6) as previously described [Brown et al., 1992]. Briefly, after development, the TLC plate was subjected to phosphorimaging (storage phosphorscreen; Kodak, Rochester, NY). The image was realised by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA) and quantitated using ImageQuant software (version 3.15). Standards (P9-derived peptides) were added to each plate prior to analysis.

### Ultraviolet Irradiation

HeLa cell monolayers or suspension cultured cells were irradiated with UVC 20 h prior to measuring peptidase activity as previously de-

scribed [Brown et al., 1993]. Briefly, the monolayer cells were washed twice with HBSS and then overlaid with 200 µl HBSS prior to irradiation from a UV source (UVS-52 Minneralite lamp; Ultraviolet Products, San Gabriel, CA) with a predominant output at 254 nm. Irradiations were performed eight wells at a time with a flux of 0.93 $Jm^{-2}s^{-1}$ for a total of 11 s (total dose 10 $Jm^{-2}$), after which HBSS was removed and the cells given fresh tissue culture media prior to assay the next day. Sham-irradiated cells were treated in a similar fashion except they were not exposed to UV irradiation.

HeLa cells in suspension culture were centrifuged (200*g* for 10 min) and washed twice with PBS (37°C) prior to being resuspended at $2.5 \times 10^7$ cells/ml in phenol red–free HBSS. These cells were plated onto petri dishes (10 cm diameter; Corning, Corning, NY) and irradiated as described above. The cells were then returned to spinner flasks (1 l) at a final concentration of $2.5 \times 10^5$ cells/ml RPMI containing 5% FBS. The cells were returned to the incubator and the membranes isolated the following day. Sham-irradiated suspension cells were treated in a similar fashion except they were not exposed to UV irradiation.

### Preparation of Membrane Fragments

The preparation of HeLa cell membrane fragments was based on a modification of the method of Harano and Mizuno [1994]. The procedure was as follows. HeLa cells ($\sim 2–5 \times 10^8$ cells) removed from the spinner flask were centrifuged (200*g* at 4°C for 5 min) and washed twice with PBS before being resuspended in disruption buffer (20 mM HEPES buffer (pH 7.3) containing 20 mM NaCl, 1 mM PMSF, and 1 µg/ml leupeptin). The cells were sonicated ($2 \times 10$ s bursts, 40% output on a Branson Sonifier 250) and then centrifuged (10,000*g* at 4°C for 10 min). The cell pellet was discarded, as it contained mainly intracellular organelles. The supernatant (containing cell membranes) was centrifuged (25,000*g* at 4°C for 30 min), and the membrane pellet resuspended in 20 mM HEPES buffer (pH 7.3) containing 0.5 M NaCl prior to being centrifuged (25,000*g* at 4°C for 30 min). The pellet was then resuspended in 20 mM HEPES buffer (pH 7.3) containing 20 mM NaCl and used as the membrane preparations in this study. Protein levels were determined by the BCA method using BSA as the standard.

### Effect of PMA on Membrane "TGFαase" Activity

Suspension HeLa cells were washed thrice with PBS before being suspended in serum-free RPMI at $2.5 \times 10^7$ cells/ml. PMA was added to the medium to give a concentration of 1 µM, and the cells were placed in a 37°C incubator and periodically shaken over a 30 min period. This time was chosen as it caused maximal enhancement of "TGFαase" activity in CHO cells [Harano and Mizuno, 1994]. After this time the cells were centrifuged (200*g* for 5 min at 4°C) and the membranes isolated as described above.

### 5′-Nucleotidase Activity

The level of 5′-nucleotidase activity present in the membrane fragments was determined using the method of Gentry and Olsson [1975]. Briefly, 50 µl of each cell fraction was added to 200 µl assay solution (60 mM Tris-HCl (pH 7.4), 5 µM AMP, and 0.1 µCi [$^{14}$C]AMP in the presence or absence of α,β-methyleneadenosine 5′-diphosphate [20 µM]) in a 1.5 ml Eppendorf tube and incubated at 37°C. After 30 min, 100 µl $ZnSO_4$ (5% w/v) and 100 µl $Ba(OH)_2$ (0.15 M) were added and the tubes spun on a microfuge (8,500*g*) for 60 s. A 200 µl aliquot was removed for liquid scintillation counting. Activity of 5′-nucleotidase was determined as those counts which were inhibited by α,β-methyleneadenosine 5′-diphosphate. The number of counts liberated was expressed as a function of protein levels. The increase in the level of enzyme enrichment was determined as the level of activity in the sample divided by the level of activity present on intact cells.

### Enzyme Inhibition Studies

The effect of the inhibitors on enzyme activity was assayed in the presence of 10 µM bestatin to eliminate confounding aminopeptidase activity. The inhibitors were added to the final concentration in the suspended membrane fragments, 10 min prior to the addition of 10 pmole $^{125}$I-P9 (final concentration 140 nM). After 30 min, a sample was spotted on a TLC plate, dried, run, developed, and quantified as described earlier. The amount of P5 formed from P9 in the presence of 10 µM bestatin was used as the control and was denoted 100%. The rate of P5 formed from P9 in the presence of the inhibitor was calculated as a percentage of that observed in the controls.

### Calculations

The picomoles of P9-derived peptides separated by TLC were quantified using the phosphorimager. The rate of P9 hydrolysis or formation of P9-derived peptides in the medium samples were expressed as a function of the cellular or membrane protein levels.

Statistical significance due to UV irradiation, FBS, bestatin, 1,10-phenanthroline, or PMA on the hydrolysis of P9 by HeLa cell monolayers was determined by the use of Student's *t*-test or Scheffé's test for multiple comparisons, where appropriate.

## RESULTS

### "TGFαase" Activity on Intact Cells

The cleavage profile of P9 by intact monolayer cells results in the formation of P1 (Y), P2 (YV), and P3 (YVA) along with very small amounts of P5 (YVAAA) (Fig. 1). P1 formation was due to the activity of an aminopeptidase, which in HeLa cells is not the ubiquitous aminopeptidase N [Brown et al., 1996] but is inhibited by the leucine aminopeptidase inhibitor bestatin [Suda et al., 1976].

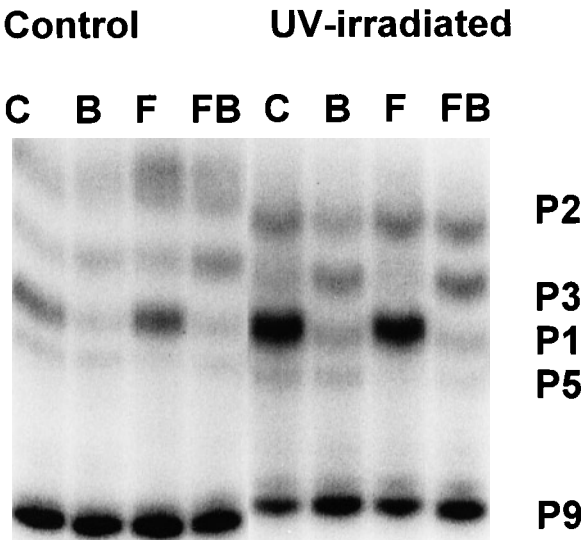


**Fig. 1.** Effect of UV on the hydrolysis of P9 by intact HeLa cell monolayers. HeLa cell monolayers were grown to semiconfluency in 24-well plates. The cells were irradiated (0 or 10 $Jm^{-2}$) 20 h prior to the commencement of the experiment as described in Materials and Methods. At the commencement of the experiment, the cells were washed twice with phenol red–free HBSS before being overlaid with 200 µl HBSS containing 10 pmoles $^{125}$I-P9 along with either nothing (*lane C,* controls), 10 µM bestatin (*lane B*), 2% FBS (*lane F*), or 2% FBS plus 10 µM bestatin (*lane FB*). After 30 min the medium was removed and 40 µl spotted onto a TLC plate, developed, and quantified as described in Materials and Methods.

The formation of P5 from P9 would result from cleavage at the bond ($A_5V_6$) which is cognate with the N-terminal cleavage site of prepro-TGFα ($A_{38}V_{39}$). The product of this candidate "TGFαase" (P5) on the HeLa cell surface was less abundant than that of the aminopeptidase (P1) but was a little enhanced when 10 µM bestatin was present in the medium, as can be seen in Figure 1 and is quantified in Table I. The product P3 behaved similarly with and without bestatin. This is due either to the fact that the aminopeptidase is more active than the "TGFαase" and the putative tripeptidyl peptidase or that it can cleave the N-terminal $^{125}$I-tyrosine from most of the products of P9 hydrolysis generated by HeLa ectoendopeptidase activity. It was noted that the ratio of P9-derived peptides obtained from HeLa cell monolayers exposed to 140 nM P9 was similar to that seen in the presence of 50 nM P9 (results not shown).

Since FBS has been shown to enhance candidate "TGFαase" activity in other cell lines [Derynck et al., 1984; Pandiella and Massagué, 1991a,b], its effects on the HeLa cell surface enzymes were examined. The rate of P9 cleavage by cell surface enzymes increased when 2% FBS was added to the assay medium (Fig. 1), the greatest increase being due to the 1.5-fold stimulation of P1 formation in sham-irradiated cultures, as seen in Table I. This was partly at the expense of the P2, P3, and P5 products, and their apparent loss could be due to the activation of the aminopeptidase in turn processing P2, P3, and P5 further to P1. However, the addition of bestatin inhibited aminopeptidase activity on the cell surface but did not significantly increase the formation of P5 from P9 in the presence or absence of 2% FBS. Notable was the inhibitory effect of FBS on the amount of P5 product formed, whether in the presence or absence of bestatin (Table I).

Dose response data were generated as seen in Figure 2 to clarify the effects of serum on the assay of P9 peptidolysis. The stimulatory effect of FBS on P9 hydrolysis is seen to be maximal (50% increase) at 2% FBS, declining nearly to serum-less levels at and above 5%. This corresponded to a twofold increase in P1 and P2 production, suggesting that the serum effects were mainly modulating ectoaminopeptidase and ectodipeptidyl peptidase activity. The pro-

**TABLE I. Comparing the Effect of Different Factors on the Rate of 10 pmol P9 Hydrolysis by HeLa Cell Monolayer Cultures Over 30 Min***

| P9-derived peptide fragment | Structure | Sham-irradiated cultures | | | | UV-irradiated cultures | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Con | Bestatin (10 μM) | FBS (2%) | FBS + bestatin | Con | Bestatin (10 μM) | FBS (2%) | FBS + bestatin |
| P2 | YV | 5.1 ± 1.3 | 4.1 ± 0.5 | 3.8 ± 1.1 | 2.4 ± 0.5 | 4.6 ± 0.6 | 6.6 ± 1.9 | 4.6 ± 3.4 | 7.3 ± 1.3[h] |
| P3 | YVA | 4.4 ± 1.6 | 6.5 ± 0.8 | 3.2 ± 1.1 | 7.7 ± 1.8 | 5.0 ± 1.1 | 7.5 ± 0.3[a] | 6.6 ± 0.9[f] | 7.8 ± 1.3 |
| P1 | Y | 12.4 ± 1.3 | 3.6 ± 0.6[d] | 18.4 ± 2.4 | 3.6 ± 0.8[d] | 28.2 ± 4.2[h] | 9.4 ± 1.9[d,g] | 32.0 ± 7.5 | 6.1 ± 0.6[d,g] |
| P5 | YVAAA | 2.6 ± 0.7 | 3.3 ± 0.5 | 0.2 ± 0.1[d] | 0.7 ± 0.3[a] | 3.5 ± 1.1 | 4.3 ± 1.1 | 0.5 ± 0.2[a] | 0.6 ± 0.1[a] |

*The effect of bestatin (10 μM) and/or FBS (2%) on the hydrolysis of 10 pmole $^{125}$I-P9 by irradiated and control HeLa cell monolayers over 30 min was measured as described in Materials and Methods. Results were expressed as picomoles peptide/milligram cell protein and represent the means ± SEM of five to ten separate experiments. The statistical significance of the difference between the peptide levels in control vs. FBS, bestatin, and FBS/bestatin groups are represented as $^{a}P < 0.05$, $^{b}P < 0.025$, $^{c}P < 0.01$, and $^{d}P < 0.005$, while the differences between assayed peptide levels of UV- and sham-irradiated cultures are represented as $^{e}P < 0.05$, $^{f}P < 0.025$, $^{g}P < 0.01$, and $^{h}P < 0.005$.




**Fig. 2.** Effect of FBS on the hydrolysis of P9 by intact HeLa cell monolayers. HeLa cell monolayers were grown to semiconfluency in 24-well plates. At the beginning of the experiment, the cells were washed twice with phenol red–free HBSS before being given 200 μl HBSS containing 10 pmoles $^{125}$I-P9 and (0–10%) FBS in the presence or absence of 10 μM bestatin. After 30 min, the medium was removed and 40 μl spotted onto a TLC plate which was developed and quantified as before. The fractions recovered were P2 (■), P3 (◆), P1 (▲), P5 (▼), and P9 (●). Those samples measured in the presence of bestatin are shown with open symbols, while for those in its absence closed symbols are used. Results are expressed as the means ± SEM of four separate experiments.

duction of P5 (presumably due to TGFαase activity) declined progressively with increasing concentrations of FBS added to the assay medium, whether in the presence or absence of bestatin. The cells were also incubated for 4 h in serum-free medium prior to assay in order to ensure that there were no serum carry-over effects when the experiments were performed. The viability of these serum-depleted cells determined by Trypan blue staining was the same as those incubated in medium containing 5% FBS, and the effects of FBS on the activity of cell surface proteases was similar in both conditions (data not shown).

The effect of 1 μM PMA on the activity of the cell surface proteases in serum-depleted cells

was tested because PMA is known to activate PKC in HeLa cells [Flint et al., 1993] and was reported to enhance "TGFαase" activity in CHO cells under similar conditions [Pandiella and Massagué, 1991a,b; Harano and Mizuno, 1994; Levine, 1994; Ramesh and Levine, 1995]. We found that neither PMA nor its carrier solvent, ethanol, enhanced the rate of P5 formation from P9 in these cells but was in fact inhibitory, as seen in Table II. This suggests that HeLa cell surface "TGFαase" activity is not stimulated by an upregulation of PKC activity.

**UV effects.** We have previously shown that UVC irradiation enhanced the level of ectopeptidase activities observed in HeLa cells [Brown et al., 1993, 1994], rising to a maximum at 16–20 h. However, the effect is not immediate (≤30 min after exposure to UVC irradiation) even in those cells exposed to 500 $Jm^{-2}$ UVC (Fig. 3). Exposure of the cells to 500 $Jm^{-2}$ UVC had no immediate (≤30 min) effect on cell viability or adherence, however; after 20 h, the adherence of cells exposed to 500 $Jm^{-2}$ UVC had fallen to 19% of controls, and the viability of these attached cells was only 51%. As previously observed (Table I), there was a statistically significant ($P < 0.005$) 2.3-fold increase in the formation of P1 from P9 in the absence of bestatin and serum in cells 20 h after receiving a low (10 $Jm^{-2}$) UVC exposure, which was insignificantly lower—1.7-fold increase in the aminopeptidase activity—when assayed in the presence of FBS (Table I).

The interpretation of data on the UV activation of "TGFαase" was made difficult using intact cells because of the interaction of several ectopeptidases, in particular the prominence of the aminopeptidase, which is readily apparent in Figure 3. We therefore repeated these experiments using plasma membrane fragments isolated from irradiated cells, simultaneously addressing the issue of the membrane localisation of "TGFαase," by a direct approach.

### Membrane Isolation

To avoid the need for exposure of monolayers to enzymatic procedures, we used suspension cultures of HeLa cells for rapid harvesting to obtain sufficient material to prepare adequate quantities of plasma membrane fragments. The cultures used had approximately 90% viable cells containing 53.4 ± 3.2 mg protein/$10^8$ cells and from which 1.12 ± 0.07 mg membrane protein/$10^8$ cells was obtained (n = 8, SEM, respectively). In the isolation procedure we found that sonication (4°C) increased the yield of membrane fragments (data not shown). The 5′-nucleotidase assay was used as a marker for the plasma membrane in the isolation procedure [Gentry and Olsson, 1975]. The specific activity of 5′-nucleotidase activity was 147-fold higher in the purified membrane fragments compared to that observed in intact cells (Table III). Most notably, as the purification proceeded, the profile of peptide products from P9 (in the presence of bestatin) changed from the prominence of P2 and P3 to that of P5 (the ratio of P5/(P2 + P3) was 0.01 in intact cells and 2.51 in the final membrane pellet), indicating a relative purification of 250-fold of the "TGFαase" from the other endopeptidases (Table III). This can be seen in Figure 4 and is quantified in Table IV. The loss of the high levels of P2 and P3 observed in the original cell suspension is apparently due to a looser association with the cell surface (Fig. 4, lanes 1–3), as the proportions of these peptides are lower when extracts of the washed cells were examined (Fig. 4, lanes 6, 8, 9) (the loss of P9 from some lanes is due to the variation in the amounts of protein (enzymes)

**TABLE II. Effect of PMA on the Hydrolysis of P9 by Serum-Depleted HeLa Cell Monolayers***

| | P9-derived peptide fragments (pmoles/mg cell protein) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | | | | +10 μM bestatin | | | |
| Medium | P2 | P3 | P1 | P5 | P2 | P3 | P1 | P5 |
| HBSS alone | 6.1 ± 0.6 | 1.9 ± 0.3 | 14.7 ± 2.5 | 2.5 ± 0.6 | 5.9 ± 0.9 | 2.9 ± 0.3[c] | 3.3 ± 0.6[e] | 3.7 ± 0.9 |
| +1 μM PMA | 5.1 ± 0.4 | 1.8 ± 0.0 | 10.3 ± 1.0 | 0.9 ± 0.1 | 8.1 ± 0.3[e] | 6.5 ± 0.3[a,e] | 4.9 ± 0.4[e] | 2.6 ± 0.4[d] |
| +0.1% EtOH | 7.0 ± 0.6 | 2.5 ± 0.2 | 8.5 ± 1.3 | 1.8 ± 0.1 | 5.1 ± 0.4[b] | 3.6 ± 0.4[d] | 4.2 ± 0.4[d] | 3.4 ± 0.7[d] |

*The effect of bestatin (10 μM) on the hydrolysis of 10 pmole $^{125}$I-P9 over 30 min was measured in HeLa cells exposed to serum-free media for 4 h. The effects of different molecules on the degradation of P9 was examined as described in Materials and Methods. Results were expressed as picomoles substrate/milligram cell protein and represents the means ± SEM of three to six separate experiments. The statistical significance of the difference between the peptide levels in control vs. PMA or ethanol-treated groups are represented as [a]$P < 0.005$, while the differences between asasyed peptide levels of control and bestatin-treated cultures are represented as [b]$P < 0.05$, [c]$P < 0.025$, [d]$P < 0.01$, and [e]$P < 0.005$.

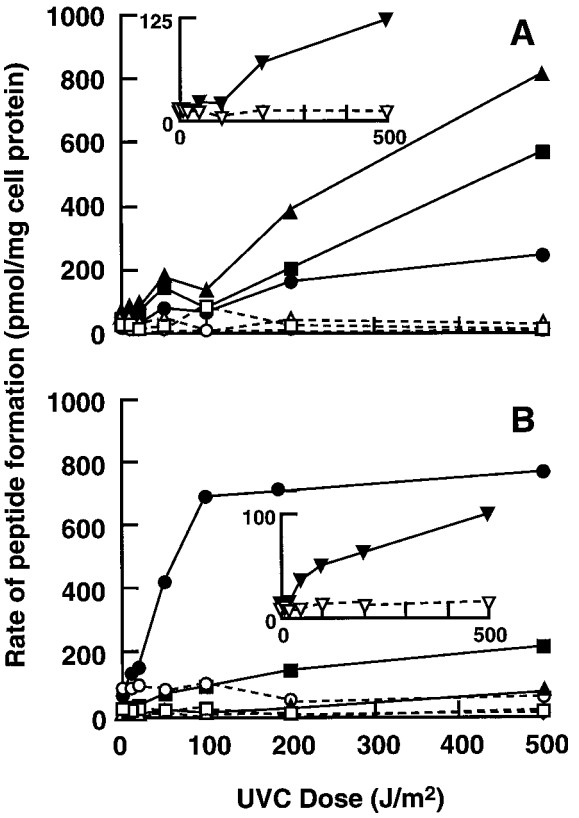


**Fig. 3.** Effect of UV on the hydrolysis of P9 by intact HeLa cells. HeLa cell monolayers were grown to semiconfluency in 24-well plates. The cells were irradiated (0–500 $Jm^{-2}$) either 30 min or 20 h prior to the commencement of the assay as described in Materials and Methods. After the indicated interval, the cells were washed twice with phenol red–free HBSS before being given 200 µl HBSS containing 10 pmoles $^{125}$I-P9 along with either (**A**) 10 µM bestatin or (**B**) no inhibitor (control). After 30 min the medium was removed and 40 µl spotted onto a TLC plate which was developed and quantified. The fractions recovered were P2 (■), P3 (▲), P1 (●), and P5 (▼). The results obtained after 30 min are shown as open symbols, while those from 20 h are expressed as closed symbols. Results are the means of three separate experiments. The profiles of P5 production are enclosed as inserts in the figure.

present and thus the degree of P9 hydrolysis). Lanes 3–5 in Figure 4 represent the different fractions of the cells after sonication. The emergence of the P5 marker for "TGFαase" activity in these sedimented membrane fractions is already clear in the supernatant from the lower speed centrifugation of the cell sonicate which still contains the plasma membranes (Fig. 4, lane 4). The differential association of P5 production with membranes becomes more obvious as the next wash is significantly enriched in P2- and P3-producing activity compared with its washed pellet (Fig. 4, lane 7 vs. 6). The further purification of membrane "TGFαase" can be seen from the peptide product profiles of the pellets after the next two washes (Fig. 4, lanes 8, 9). The increase in the ratio of ("TGFαase")/(dipeptidyl and tripeptidyl peptidases) in the various steps of the membrane fractionation can be seen in Tables III and IV, whereas the ratio of 5′-nucleotidase/"TGFαase" activities remained constant at 22.1 ± 1.3 (n = 3). These membrane fragments (Fig. 4, lane 9) were used in subsequent assays.

**Membrane "TGFαase" activity and inhibition.** The cleavage of P9 by the membrane proteases was carried out in the presence of the leucine aminopeptidase inhibitor bestatin [Suda et al., 1976], because aminopeptidases may eliminate some of the P5 formed from P9, resulting in an underestimate of the rate of P5 production, as seen above (e.g., Tables I, III). Figure 5 shows that 10 µM bestatin gave significant inhibition (46%) of aminopeptidase activity, but increase of inhibitor concentration to 100 µM produces only a further 18% inhibition (total 64%). The inhibitory effect of bestatin on the aminopeptidase activity of the HeLa cell membrane fragments was similar to that observed on the aminopeptidase activity of intact L5178Y cells [Leyhausen et al., 1983]. The rate of P5 formation from 140 nM P9 by the membrane fragments over 30 min was linear, as can be seen in Figures 6 and 7. The apparent initial rates of the "TGFαase" responsible for this cleavage were 5.6 and 4.9 pmole/min/mg membrane protein (n = 6), respectively, in the two different membrane preparations used in Figures 6 and 7.

The addition of 0.1% Brij-35 to the membrane fragments caused a 20% reduction in the rate of P5 formation from P9. Sonication of the membrane fragments resulted in a 30% reduction in activity, yet when the membranes were sonicated in the presence of detergent the effects were not additive (only a 30% reduction). There was no difference in enzyme activity if Brij-35 was added before or after the membranes were sonicated. Similarly, when the membrane fragments were sonicated in the presence of 10 µM Zinc acetate or 0.1% Triton X-100 or a combination of both, there was a reduction in the rate of P5 formation from P9, as seen in Figure 7. $Zn^{2+}$ caused a 26% inhibition, while with Triton X-100 it was 55%, but again the effects were not additive (combined inhibition 56%).

We have shown earlier, using intact adherent HeLa monolayers, that 1 µM PMA did not activate the "TGFαase" (see Table II). Since 1 µM

**TABLE III. Effect of UV on 5′-Nucleotidase Activity and the Ratio of P5/(P2 + P3) Formation in HeLa Cell Membrane Fragments***

| Membrane fragment | Sham-irradiated cultures | | | UV-irradiated cultures | | |
|---|---|---|---|---|---|---|
| | Relative 5′-nucleotidase activity | Rate P5 formation (pmol/mg per min) | Ratio P5/(P2 + P3) | Relative 5′-nucleotidase activity | Rate P5 formation (pmol/mg per min) | Ratio P5/(P2 + P3) |
| Cells | 1 | | 0.01 ± 0.01 | 1 | | 0.04 ± 0.01 |
| Sonicate | 2.7 ± 0.8 | | 0.07 ± 0.01 | 1.1 ± 0.4 | | 0.11 ± 0.02 |
| Low speed supernatant | 12 ± 2 | | 0.12 ± 0.02 | 11 ± 2 | | 0.22 ± 0.03 |
| First high speed spin pellet | 17 ± 3 | 0.8 ± 0.1 | 0.43 ± 0.08 | 13 ± 2 | 1.4 ± 0.3 | 0.71 ± 0.16 |
| Second high speed spin pellet | 98 ± 16 | 4.7 ± 0.2 | 2.1 ± 0.2 | 84 ± 13 | 10 ± 1 | 1.5 ± 0.1 |
| Final membrane pellet | 147 ± 18 | 5.9 ± 0.8 | 2.5 ± 0.3 | 125 ± 21 | 15 ± 1 | 1.9 ± 0.3 |

*HeLa cell membranes were prepared and the level of 5′-nucleotidase activity and formation of P5, P3, and P2 were determined as described in Materials and Methods. The level of 5′-nucleotidase activity/milligram cell protein in intact cells was normalised, with intact cells representing 1.0 and that of the various fractions compared therewith. Results are the means ± SEM of three to six separate determinations, assayed in the presence of bestatin.

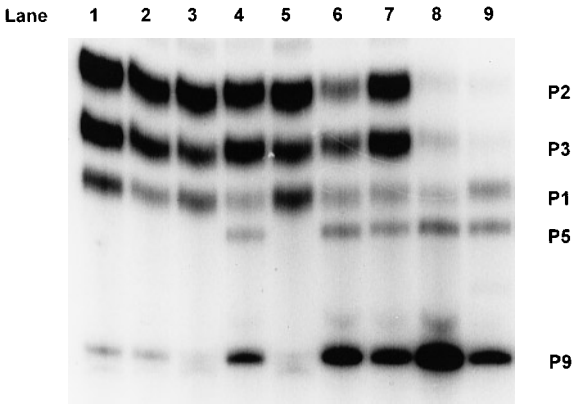


**Fig. 4.** The hydrolysis of P9 by HeLa cell fractions from the plasma membrane isolation protocol. Membranes were isolated from HeLa cells grown in suspension as described in Materials and Methods. Samples (50 µl) from different steps in the isolation procedure were incubated with 20 µl HBSS containing 10 pmoles $^{125}$I-P9 plus 10 µM bestatin for 30 min. At the end of this period, samples were spotted onto a TLC plate, developed, and quantified as before. Lanes represent a phosphorimage of a TLC separation of the products of P9 hydrolysis in assays using the (*lane 1*) original cell pellet, (*lane 2*) PBS-washed cell pellet, (*lane 3*) cell sonicate, (*lane 4*) supernatant after 10,000*g* spin, (*lane 5*) pellet after 10,000*g* spin, (*lane 6*) pellet after first 25,000*g* spin, (*lane 7*) supernatant after 25,000*g* spin, (*lane 8*) pellet after 0.5 M NaCl wash, and (*lane 9*) final membrane pellet. The quantified data, after correcting for total volume and protein content, are recorded in Table IV.

PMA caused maximal activation of the putative "TGFαase" in CHO cells [Harano and Mizuno, 1994], HeLa cells in suspension culture were exposed to this concentration before the membrane fragments were prepared. PMA had no effect on isolated membrane "TGFαase" activity (data not shown), confirming our earlier observation that this enzyme on HeLa cells does not appear to be under direct regulation by PKC activity.

**UV effects on membrane "TGFαase" activity.** When the HeLa cell suspension cultures were irradiated (10 $Jm^{-2}$) and harvested 20 h later, we observed that cell viability (Trypan blue exclusion) fell from 90% to 60%. Even though viability fell, the level of cell protein (54.0 ± 6.5 mg/$10^8$ cells, n = 8, SEM), the amount of recovered membrane protein (1.07 ± 0.06 mg/$10^8$ cell, n = 8, SEM), and the level of 5′-nucleotidase in the plasma membranes prepared from these cells were similar to those seen in sham-irradiated controls (Table III). The time course of P5 formation from P9 by plasma membranes isolated from UV-irradiated cells can be seen in Figure 6. The rate of P5 formation from 140 nM P9 was 13.8 pmol/min/mg membrane protein (n = 4), a rate which was 2.5-fold greater than that observed in control membranes, and was of similar magnitude to that seen previously for the ectoendopeptidase in intact cells [Brown et al., 1993, 1994]. The relative increase in UV-induced membrane TGFαase activity to that of 5′-nucleotidase was also similar (2.4-fold), as seen in Table III. As was seen with plasma membranes isolated from control cells (Fig. 7), sonication of these membrane fragments from UV-irradiated cells even in the presence of 10 µM Zinc acetate and/or 0.1% Triton X-100 detergent resulted in a simi-

**TABLE IV. Comparison of the Patterns of P9 Hydrolysis by HeLa Cell Membranes at Various Stages of the Isolation Procedure***

| Lane | Fraction | Total protein (mg) | Rate of peptide formation (pmole/mg protein per min) | | | | Ratio |
|---|---|---|---|---|---|---|---|
| | | | P2 | P3 | P1 | P5 | P5/(P2 + P3) |
| 1 | Original cell pellet | 292 | 1.8 | 1.3 | 0.6 | — | 0 |
| 2 | PBS-washed cell pellet | 283 | 2.3 | 1.8 | 0.6 | — | 0 |
| 3 | Cell sonicate | 276 | 2.1 | 1.0 | 0.6 | — | 0 |
| 4 | Supernatant after 10,000*g* spin | 38.4 | 2.2 | 2.0 | 0.3 | 0.2 | 0.05 |
| 5 | Pellet after 10,000*g* spin | 245 | 4.0 | 2.0 | 1.6 | — | 0 |
| 6 | Pellet after first 25,000*g* spin | 9.8 | 1.1 | 1.5 | 0.5 | 0.5 | 0.19 |
| 7 | Supernatant after first 25,000*g* spin | 26.3 | 6.7 | 6.1 | 0.9 | 0.7 | 0.05 |
| 8 | Pellet after 0.5 M NaCl wash | 5.8 | 0.7 | 1.5 | 1.5 | 3.9 | 1.77 |
| 9 | Final membrane pellet | 5.7 | 0.9 | 1.0 | 5.5 | 5.0 | 2.63 |

*Membrane fractions from HeLa cell controls were prepared as described in Materials and Methods. Briefly, the cells were sonicated and then centrifuged at 10,000*g* for 10 min and the supernatant (containing membranes) recentrifuged at 25,000*g* for 30 min. The pellet was resuspended in a Hepes buffer and centrifuged at 25,000*g* for 30 min. The pellet was then washed with a Hepes buffer containing 0.5 M NaCl and centrifuged (25,000*g* for 30 min) with the membranes suspended in Hepes buffer and used in other assays. Bestatin (10 μM) and 10 pmole $^{125}$I-P9 was added to 50 μl membrane fractions. After 30 min, samples were removed and spotted onto a TLC plate and quantified as described in Materials and Methods. Results expressed are from that of an isolation experiment using $4.5 \times 10^8$ cells. —, not detected.

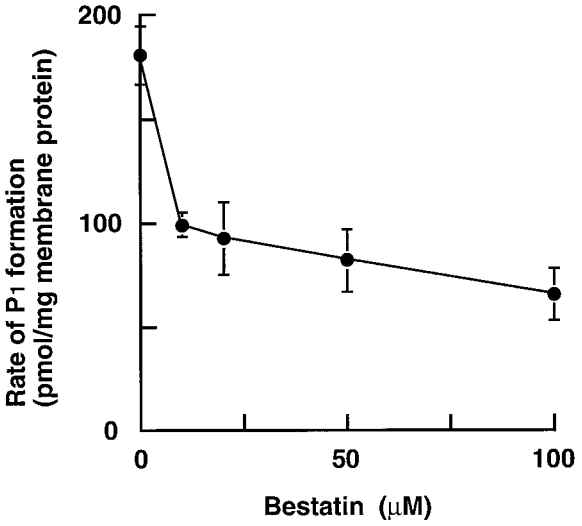


**Fig. 5.** Effect of bestatin on aminopeptidase activity in HeLa cell membrane fractions. Membranes were isolated from HeLa cells grown in suspension as described in Materials and Methods. The fragments (50 μl) were incubated with 20 μl HBSS containing 10 pmoles $^{125}$I-P9 plus 0–100 μM bestatin for 30 min. At the end of this period, samples were spotted onto a TLC plate, developed, and quantified as described. Results are the means ± SEM of four separate experiments.

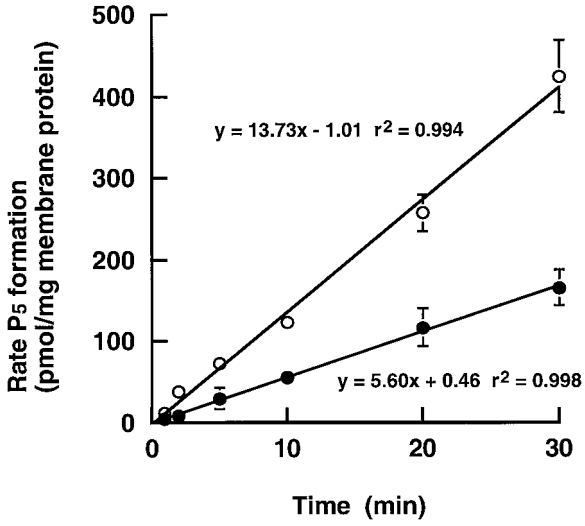


**Fig. 6.** The effect of UVC on the rate of P9 cleavage by HeLa cell membrane fragments. Membranes were isolated from control (●) and UVC-irradiated (○) HeLa cells grown in suspension as described in Materials and Methods. The fragments (50 μl) were incubated with 20 μl HBSS containing 10 pmoles $^{125}$I-P9 plus 10 μM bestatin over 30 min. At various times, samples were spotted onto a TLC plate, developed, and quantified as described. Results are the means ± SEM of four to six separate experiments.

lar decrease of "TGFαase" activity (data not shown).

### Inhibitor Profiles of the "TGFαase"

The inhibitory effect of serine protease and metalloprotease inhibitors on the formation of P5 from P9 by the "TGFαase" in HeLa cell membrane preparations is seen in Table V. The serine and elastase protease inhibitors (PMSF, leupeptin, and elastatinal) had no effect on "TGFαase" activity, while the metalloenzyme inhibitors (1,10-phenanthroline, EDTA, and EGTA) were inhibitory. Amastatin, like bestatin, is inhibitory towards leucine aminopep-

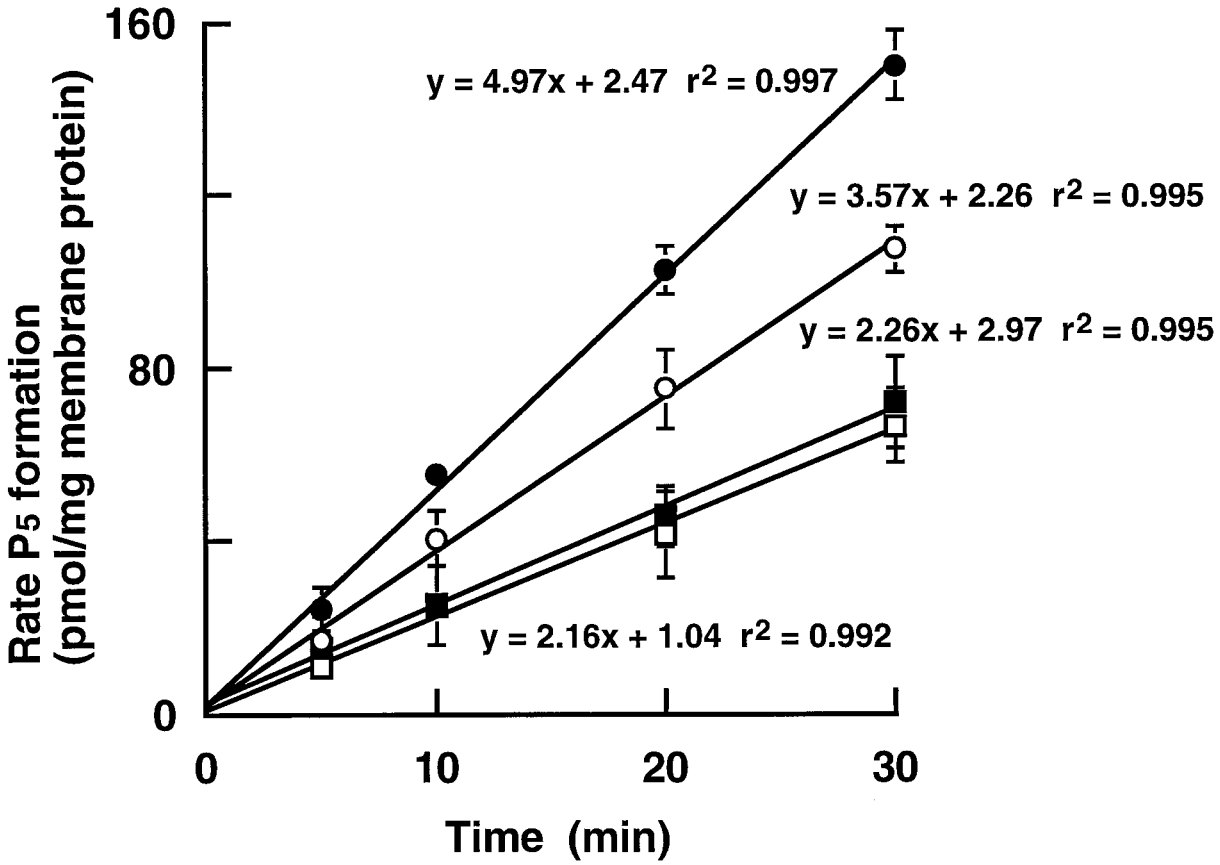


**Fig. 7.** Effect of detergent and/or zinc on the cleavage of P9 by HeLa cell membrane fragments. Membranes were isolated from suspension cultured HeLa cells as described in Materials and Methods. The membrane fragments were sonicated (2 × 15 s bursts) in the presence of nothing (controls) (●), 10 μM Zinc acetate (○), 0.1% Triton X-100 (■), and 0.1% Triton X-100 plus 10 μM Zinc acetate (□). The fragments (50 μl) were incubated with 20 μl HBSS containing 10 pmoles $^{125}$I-P9 plus 10 μM bestatin over 30 min. At various times, samples were spotted onto a TLC plate, developed, and quantified as described. Results are the means ± SEM of four separate experiments.

tidase and aminopeptidase N and W but does not inhibit aminopeptidase B activity [Tieku and Hooper, 1992]. A similar result was observed in the membrane fragments isolated from UVC-irradiated cells.

### Effect of Metalloprotease Inhibitors on "TGFαase" Activity in Intact HeLa Cell Monolayers

Since the "TGFαase" of HeLa cell membranes was shown to be inhibited by metalloprotease inhibitors (Table V), we examined the effect of 1 mM 1,10-phenanthroline on the hydrolysis of P9 by control and UV-irradiated (10 $Jm^{-2}$) HeLa cell monolayers. Table VI shows that 1,10-phenanthroline significantly inhibited both aminopeptidase and "TGFαase" activity in control and UV-irradiated cell monolayers. This result agrees with that seen earlier using membrane fragments and suggests that the HeLa cell "TGFαase" and the aminopeptidase are metalloenzymes.

## DISCUSSION

The protease(s) believed responsible for cleaving preproTGFα to TGFα resides on the cell membrane [Mueller et al., 1990; Pandiella and Massagué, 1991a,b; Bosenberg et al., 1992; Brown et al., 1993; Harano and Mizuno, 1994; Lee et al., 1995]. There is debate in the literature as to the exact nature of these protease(s) and on their role in the processing of TGFα from its precursor. This confusion arises from the fact that, apart from a few studies using intact cells [Pandiella and Massagué, 1991a,b; Pandiella et al., 1992; Ramesh and Levine, 1995], no one has used preproTGFα directly as a substrate to isolate the enzyme(s) responsible for its processing. Many laboratories have used Suc-Ala-Ala-Ala-MCA to measure enzyme activ-

**TABLE V. Effect of Protease Inhibitors on the P5 Hydrolase Activity Observed in Control and UV-Irradiated HeLa Cell Membranes Compared to That Observed in Other Cell Lines***

| Inhibitor (concentration) | Controls | UV- irradiated cells | CHO cells [Harano and Mizuno, 1994] | HCT116 cells [Levine, 1994] | GEO cells [Levine, 1994] |
|---|---|---|---|---|---|
| Control (bestatin 10 μM) | 100 | 100 | 100 | 100 | 100 |
| PMSF (1 mM) | 98 ± 5 | 105 ± 3 | 93 | 1 | 1 |
| Leupeptin (100 μM) | 94 ± 2 | N.D. | 74 | | |
| Elastatinal (20 μM) | 100 ± 5 | 107 ± 3 | 101 | 1 | 1 |
| EDTA (1 mM) | 81 ± 4 | 91 ± 4 | 113 | | |
| EDTA (10 mM) | 37[2] | 30[2] | | | |
| EGTA (1 mM) | 89 ± 5 | 87 ± 5 | 100 | | |
| EGTA (10 mM) | 49[2] | 26[2] | | | |
| Phenanthroline (0.25 mM) | 61 ± 1 | N.D. | | | |
| Phenanthroline (1 mM) | 19[2] | 21[2] | 100 | | |
| Amastatin (2 mM) | 104 ± 4 | N.D. | | | |

*Inhibitors (at the final concentrations listed) and bestatin (10 μM) were added to the membrane fractions from control and UV-irradiated HeLa cells for 10 min prior to the addition of 10 pmole $^{125}$I-P9. After 30 min, samples were removed and spotted onto a TLC plate, and the proportion of P5 produced was quantified as described in Materials and Methods. All assays were conducted in the presence of 10 μM bestatin to reduce the effect which aminopeptidases have on the hydrolysis of P9 and its products. The amount of P5 liberated from P9 in the presence of bestatin alone with either the sham-irradiated or the UV-irradiated set was used as the control value (100%) for the respective series and the amounts of P5 liberated in the presence of the indicated inhibitor normalised to these. Results are expressed as the means ± SEM of four to six separate experiments. The P5 hydrolase activity of the UV-irradiated cell membrane preparation was 2.5-fold greater than the sham-irradiated (control) preparations. N.D., not done.
[a]Represents only one experimental value.

**TABLE VI. Comparing the Effect of 1,10-Phenanthroline on the Rate of 10 pmole P9 Hydrolysis by HeLa Cell Monolayer Cultures over 30 Min***

| | Sham-irradiated cultures | | UV-irradiated cultures | |
|---|---|---|---|---|
| P9-derived peptide | Con | Bestatin | Con | Bestatin |
| 0 mM 1,10-phenanthroline | | | | |
| P2 | 5.4 ± 0.9 | 5.3 ± 0.7 | 5.3 ± 0.8 | 6.5 ± 1.3 |
| P3 | 4.0 ± 0.4 | 6.1 ± 0.9 | 2.2 ± 0.4 | 8.1 ± 1.2 |
| P1 | 11.1 ± 1.3 | 3.4 ± 0.4 | 28.8 ± 2.3[e] | 8.8 ± 0.9[e] |
| P5 | 2.4 ± 0.2 | 2.7 ± 0.4 | 3.8 ± 0.5[c] | 4.4 ± 0.5[e] |
| 1 mM 1,10-phenanthroline | | | | |
| P2 | 7.8 ± 1.2 | 6.7 ± 1.0 | 7.8 ± 1.6 | 8.1 ± 1.9 |
| P3 | 3.3 ± 0.7 | 2.4 ± 0.3[a] | 2.4 ± 0.4 | 6.7 ± 0.8[e] |
| P1 | 2.3 ± 0.4[b] | 1.1 ± 0.3[b] | 1.4 ± 0.3[b] | 2.1 ± 0.2[b,d] |
| P5 | —[b] | —[b] | —[b] | —[b] |

*The effect of bestatin (10 μM) and/or 1,10-phenanthroline (1 mM) on the hydrolysis of 10 pmole $^{125}$I-P9 by irradiated and control HeLa cell monolayers over 30 min was measured as described in Materials and Methods. Results were expressed as picomoles peptide/milligram cell protein and represent the means ± SEM of three separate experiments. The statistical significance of the differences due to 1,10-phenanthroline inhibition are represented as [a]$P < 0.01$ and [b]$P < 0.005$, while the differences between assayed peptide levels of UV- and sham-irradiated cultures are represented as [c]$P < 0.05$, [d]$P < 0.025$ and [e]$P < 0.005$. —, not detected.

ity, while others have observed TGFα release from intact cell assays. A disadvantage in using Suc-Ala-Ala-Ala-MCA is that it lacks the scissile bond between alanine and valine which is specifically recognised during the processing of preproTGFα. The cleavage sites of preproTGFα are between $A_{39}$†$V_{40}$ on the N-terminal region and between $A_{89}$†$V_{90}$ on the C-terminal region. Proteases which cleave peptides possessing alanine at the cleavage site will also cleave this substrate liberating the fluorogenic product MCA which could give rise to an erroneous assumption that "TGFαase" activity is present. While "TGFαase" may process this molecule,

there exists a strong possibility that a tripeptidyl peptidase–like activity may also cleave this substrate which would make the interpretation of this data difficult. It was because of this problem that we have used the nonapeptide substrate corresponding to the eight amino acids which flank the N-terminal site of preproTGFα ($V_{36}A_{37}A_{38}A_{39}\dagger V_{40}\ V_{41}S_{42}H_{43}$), supplemented with an N-terminal $^{125}$I-Y for ease of analysis [Brown et al., 1992]. The formation of the pentapeptide $^{125}$I-YVAAA (denoted P5) would represent the cleavage product if "TGFαase" processed P9. A similar strategy was used to isolate enzymes which cleave the β-amyloid protein in brain tissue [Mohler et al., 1994]. We intend examining whether the C-terminal cleavage site is processed by the same enzyme which cleaves P9, as well as confirming scissile bond specificity using preproTGFα as the substrate.

It is expected that the "TGFαase" would occur on the external face of the cell membrane as cleavage of the membrane-bound preproTGFα releases TGFα from the cell into the extracellular domain [Massagué, 1990; Derynck, 1992; Lee et al., 1995]. It has been suggested, therefore, that there must be an inside-to-outside signal transducing system, which can transfer the intracellular stimulatory signal (e.g., PKC activation by PMA) to extracellular proteolytic events, across the plasma membrane [e.g. Harano and Mizuno, 1994]. For human colon carcinoma cells it has been suggested that the $Ca^{2+}$-dependent PKC-β plays a role in the processing of preproTGFα by activating the protease(s) involved [Levine, 1994; Ramesh and Levine, 1995], while, in CHO cells, activation occurred via a $Ca^{2+}$-independent PKC mechanism [Bosenberg et al., 1993]. Activation of cleavage of preproTGFα in CHO cells depends on a C-terminal valine residue in the cytoplasmic domain of preproTGFα [Bosenberg et al., 1992] which suggests the existence of a molecule that recognises the intracellular C-terminal valine and is involved in activating proteolysis, an event which occurs outside the cell. It has been proposed that two proteins, p86 and p106, are involved in this process [Shum et al., 1994].

We did not observe an immediate activation of the "TGFαase" in either intact cells or membranes from HeLa cells which had been treated with PMA or serum (Table II; Fig. 2) or with low or high fluences of UV (Fig. 3), so that the enzyme in HeLa cells is presumably not activated through changes in PKC activity, and is thus different from the TGFαase activity observed in CHO cells [Bosenberg et al., 1992; Harano and Mizuno, 1994; Levine, 1994; Ramesh and Levine, 1995]. Even though UV exposure has been shown to increase PKC activity in A431 and HeLa cells, which in turn reduced EGF and TGFα binding to the EGF receptor [Matsui et al., 1989; Matsui and DeLeo, 1990; Warmuth et al., 1994], a role for PKC in the downregulation of EGF receptor activity after UV irradiation has been questioned [Brooks et al., 1990]. Furthermore, UV irradiation increased TGFα release from various cell types and in addition augmented the number of EGF receptors on the HeLa cell surface [Ellem et al., 1988; Ley and Ellem, 1992] but not until at least 12 h after exposure. In a recent study the activation of "TGFαase" activity lagged 6 h behind aminopeptidase activation and was maximal only 20 h after UVC exposure [Brown et al., 1993], and the lack of PMA and serum upregulation of this enzyme also made the involvement of PKC unlikely (Figs. 1, 6).

The "TGFαase" in HeLa cells appears to be a metalloenzyme since EDTA, EGTA, and 1,10-phenanthroline inhibited activity while PMSF, leupeptin, or elastinal did not (Tables V, VI). The observation that "TGFαase" is a metalloenzyme differs from that seen in other studies with different cell types [Mueller et al., 1990; Pandiella and Massagué, 1991a,b; Pandiella et al., 1992; Cappelluti et al., 1993; Harano and Mizuno, 1994; Levine, 1994; Ramesh and Levine, 1995]. Metalloenzymes have been observed on cell membranes and have been shown to be involved in the cleavage and processing of many different growth factors [Ehlers and Riordan, 1991; Mohler et al., 1994; Xu et al., 1994]. The "TGFαase" seen in CHO cell membranes was inhibited by the serine protease inhibitor, DCI, and, like the HeLa cell enzyme, was not inhibited by elastatinal or PMSF. However, the HeLa cell enzyme was inhibited by metal chelators, which was not observed with CHO cells [Pandiella et al., 1992; Harano and Mizuno, 1994] or human colon carcinoma cells [Levine, 1994; Ramesh and Levine, 1995]. Other differences between the "TGFαase" activity seen in these various cell lines are recorded in Table V. In particular, TGFα cleavage from its precursor in HCT116 and GEO cells was shown to be inhibited by elastase (elastatinal) and serine protease inhibitors (PMSF) which thus differed

from the activity present in CHO and HeLa cells.

We used membrane fragments to confirm that the effect of UVC was on plasma membrane "TGFαase" activity. The membranes were prepared using the method of Harano and Mizuno [1994] except that the cells were lysed by sonication and not by homogenisation. Sonication was found to cause greater disruption of the cells, and, by centrifugation of the sonicate at 10,000*g* for 10 min, cellular debris and organelles were removed, thereby reducing contamination of the membrane preparations. The method employed by Harano and Mizuno [1994] did not mention a low speed spin step in the preparation procedure, and this may have resulted in the contamination of the membranes with intracellular particulates containing proteases, thus confounding their assays for ectoproteases. By washing the membranes several times in HEPES buffer containing different NaCl concentrations, we observed that many loosely bound proteases were removed from the membrane fragments (Table IV; Fig. 4), enabling better quantification of the "TGFαase" bound thereto.

"TGFαase" activity was increased in the membranes of UVC-irradiated cells (Fig. 6) some 2.5-fold in agreement with earlier studies on intact cells [Ellem et al., 1988; Chenevix-Trench et al., 1992; Brown et al., 1992]. The increase in "TGFαase" activity observed after UV exposure is posttranslational, as it was not due to increased mRNA or protein synthesis [Chenevix-Trench et al., 1992]. It is possible that the "TGFαase" is stored in vesicles within the cytoplasm and after receiving a stimulus these enzymes are transported to the cell membrane, thereby increasing the rate of prepro-TGFα processing. There were no obvious differences in the number or level of proteins in the membranes of UV-irradiated cells seen by silver-stained polyacrylamide gel electrophoresis gels of membrane preparations (data not shown), but the amount of enzyme could well be below detection by this method. Evidence for such a mechanism has been observed in the upregulation of glucose transporters in response to insulin binding to the cell membrane [Hirshman et al., 1990], but until antibodies are available to positively identify the plasma membrane "TGFαase" the large amounts of confounding endopeptidase activities present intracellularly [Brown et al., 1993] prevent a direct answer to shift of the "TGFαase" from intracellular pools to the plasma membrane.

Alternatively, the increase in "TGFαase" activity could be due to a modification of the lipids or proteins surrounding the enzyme which could affect the enzyme's $K_m$. UV irradiation has been shown to peroxidate membrane lipids [Hruza and Pentland, 1993; Kolling et al., 1994], and such changes in the structure of the lipids may affect the protein-lipid interaction in a fashion which could increase its activity. Furthermore, sonication of the membrane fragments in the presence of detergent resulted in a 50% loss of "TGFαase" activity (Fig. 7), suggesting the "TGFαase" activity may be modulated by a co-factor or protein or the lipid surrounding the enzyme. Bosenberg et al. [1992] postulated that another molecule is involved in maintaining "TGFαase" activity in cell membranes, and it is possible that loss of inhibitory molecules with time could also allow augmentation of the "TGFαase" activity. We plan to examine which of these factors may cause the increase in "TGFαase" activity observed in UV-irradiated cells.

## ACKNOWLEDGMENTS

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