Journal of Cellular Biochemistry 48:411–423 (1992)

Development of a Sensitive Peptidase Assay: In Search of Cell Associated Proteases Responsible for the Cleavage of preproTGF$_\alpha$

S.B. Brown, D. Krause, E. Townsend, and K.A.O. Ellem

Queensland Cancer Fund Research Unit, Joint Experimental Oncology Program, Queensland Institute of Medical Research, The Bancroft Centre, Brisbane, Queensland 4029, Australia

Abstract A radiometric assay has been developed for the detection of proteolytic activity capable of releasing transforming growth factor alpha (TGF$_\alpha$) from its membrane bound precursor. The assay is dependent upon the separation by thin layer chromatography of hydrolytic products of a nonapeptide substrate containing a radioactive iodinated tyrosine residue as a reporting group N-terminal to an octapeptide which is cognate to the N-terminal cleavage sequence of TGF$_\alpha$. We describe the selectivity of the peptidase assay with commercially purified proteases and with cell-associated peptidases, its exquisite sensitivity, and its applicability to defining peptidase activity, which may be responsible for the processing of the membrane-bound preproTGF $_\alpha$. The activity of two different elastases had different profiles which thus may be of use in characterizing them. The characteristics of the intact and extracted HeLa cell assay with respect to time, cell density, and peptidase concentration are defined, as are conditions needed to remove endogenous, confounding, proteolytic activity from the serum used to support cell culture. Intact HeLa cell cultures exhibit both exo- and endo-peptidase activity at approximately equal levels in both sparse and dense monolayer culture without relationship to cell density, and at a level equal to 1–2% of total cell activity of these enzyme classes.

Key words: cognate peptide substrate, preprotein processing, preproTGF$_\alpha$, HeLa cells, cell surface proteases, aminopeptidases, endopeptidases, product profiling, thin layer chromatography, factor regulation

Post-translational activation of many growth factors involves the proteolytic cleavage of a less active precursor. Mature transforming growth factor (TGF$_\alpha$) is released from its membrane-bound precursor via hydrolysis of peptide bonds between an alanine (A) and a valine (V) set in the hydrophobic sequences VAAA†VVSH and LLA†VVAA (Derynck et al., 1984; Lee et al., 1985). Proteolytic cleavage in an extended hydrophobic sequence is suggestive of an elastase-like or thermolysin-like cleavage (Geneste and Bender, 1969). While it was shown that porcine pancreatic elastase and polymorphonuclear leukocytic elastase were able to cleave TGF$_\alpha$ from its precursor (Ignotz et al., 1986; Mueller et al., 1990), it was not established whether the AV bonds were specifically cleaved. Site-directed mutagenesis against the scissile bond (A†VV) still resulted in the detection of mature TGF$_\alpha$, but at much reduced levels, and it was not until residual AA or AV dipeptides were removed that processing was fully blocked (Wong et al., 1989; Brachmann et al., 1989).

Many protease substrates contain chromophoric or fluorophoric groups which are directly hydrolyzed from peptide carriers via ester hydrolysis, indicative of esterolytic activity. The release of these chromophores is then monitored directly or indirectly by a change in absorbance or fluorescence. Nitroanilides, aminomethylcoumarins, and methoxynapthylamines, to name a few, have all been used extensively as leaving groups in the development of sensitive assays (cf. Castillo et al., 1979). It could be argued, however, that these chromogenic substrates are not specific or selective in that they ignore the contribution imparted to catalysis by interactions at remote subsites (Knight, 1977). These interactions can be shown to influence

Received November 15, 1991; accepted December 3, 1991.

Address reprint requests to K.A.O. Ellem, Queensland Cancer Fund Research Unit, Joint Experimental Oncology Program, Queensland Institute of Medical Research, The Bancroft Centre, 300 Herston Road, Brisbane, Queensland 4029, Australia.

catalysis such as is observed for the acid proteases (Moore et al., 1989), various metalloendoproteases (Powers and Harper, 1986), and elastases (Bode et al., 1989). Furthermore, the presence of a chromophoric leaving group may result in distorted specificity (Atlas and Berger, 1972) and proton inventory studies suggest that the catalytic mechanism for monomeric or dipeptide carrier substrates is distinct from that of extended peptides (Stein et al., 1987). While the usefulness of these synthetic substrates, which have well-defined chemical structures, in determining catalytic parameters or in the purification of enzyme(s) is well attested, their use to identify an enzyme with tight substrate specificity may be inadequate. This must be considered when designing an artificial assay for a catalytic event involved in a physiological process.

We have set out to characterize the cellular protease(s) which may be responsible for controlling the release of TGF_α. To ensure detection of a specific enzyme(s) an assay was developed in which the substrate was a nonapeptide containing an octapeptide cognate with the N-terminal cleavage sequence of TGF_α. The N-terminal octapeptide was preferred to the C-terminal octapeptide because the former contained a serine-histidine pair which aids the separation of products and substrate by TLC. We describe the characteristics of the peptidase assay, its exquisite sensitivity, and its use to define several peptidases which are associated with intact HeLa cell cultures, as a prerequisite to understanding the release of mature TGF_α (Ellem et al., 1988) during the cellular response to UV-irradiation.

MATERIALS AND METHODS

Materials

α-Chymotrypsin (TLCK treated, Type VIII), leucine aminopeptidase (Type III-CP), human leukocytic elastase, porcine pancreatic elastase (Type IV), polyoxyethylene(23)lauryl ether (Brij-35), Tris(hydroxymethyl)aminomethane (Tris), L-tyrosine, bestatin, trypsin (TPCK treated, Type XIII) were purchased from Sigma (St. Louis, MO); aminopeptidase-M, Calbiochem Corporation (Alexandria, NSW, Australia); dispase (Type II), Boehringer Mannheim Australia (Castle Hill, NSW, Australia); sodium dihydrogen orthophosphate, AJAX Chemicals Pty Ltd (Auburn, NSW, Australia); butan-1-ol and sodium chloride (NaCl), May & Baker Ltd (Dagenham, England); chloramine-T, polyoxyethylene(20) sorbitan mono-oleate (Tween-80), BDH Chemicals Ltd (Poole, England); RPMI-1640 growth media, Gibco Laboratories (Grand Island, NY); $Suc(Ala)_3NA$, Bachem Inc. (Torrance, CA); trypsin, penicillin, and streptomycin, Commonwealth Serum Laboratories (Melbourne, Victoria, Australia). All other reagents were of AR grade. All aqueous solvents were prepared with Milli-Q purified water.

Cell Culture

The epithelial cervical carcinoma cell line, HeLa, was cultured in RPMI-1640 containing penicillin (100 μg/mL), streptomycin (100 μg/mL), and 10% foetal bovine serum (FBS), heat inactivated at either 56°C for 30 min (S56) or 70°C for 60 min (S70). Cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. HeLa cells that had been selected for over 20 passages in 70°C heat inactivated serum (S70) media, from an original cell line (HeLaN56) maintained in 56°C treated serum (S56) media, are referred to as HeLaS70. Those which were not fully selected (only 1–3 passages) are referred to as HeLaN70. HeLa cell lines were shown to be mycoplasma free (Hoechst stain) (Chen, 1977).

Continuous growth of HeLa cell lines was achieved by trypsinizing and passaging (1:10 or 1:20) when near confluence. Typically 2×10^5, 10^6, and 10^7 cells were used to inoculate 2-, 20-, and 120-cm^2 petri dishes/flasks containing 1, 5, and 25 mL of fresh medium respectively, unless otherwise stated. Neutralization of the trypsin was effected by the serum. Total cell counts and viable cell number were determined on cell suspensions containing 0.1% trypan blue with a minimum of eight counts per sample (four per chamber).

Peptide Syntheses

The peptide substrate H_2N-YVAAAVVSH-NH_2, (P_9), and all possible proteolytic fragments containing the amino terminal residue tyrosine (Table I) were prepared and purified within this institute by Dr. Graham Jones and associates. Peptides were synthesized using the simultaneous multiple peptide synthesis technique originally described by Houghten (1985), utilizing benzhydrylamine resins for amide and phenylacetamidomethyl resins for acid-derived peptides upon hydrogen fluoride cleavage. The crude pep-

TABLE I. TLC Mobilities of Synthetic Peptides

Peptide	Structure	R_f BAW[a]	R_f BAWP[b]
P_1	H_2N-Y-OH	0.59	0.47
P_2	H_2N-YV-OH	0.70	0.65
P_3	H_2N-YVA-OH	0.69	0.52
P_4	H_2N-YVAA-OH	0.57	0.37
P_5	H_2N-YVAAA-OH	0.51	0.39
P_6	H_2N-YVAAAV-OH	0.60	0.40
P_7	H_2N-YVAAAVV-OH	0.67	0.52
P_8	H_2N-YVAAAVVS-OH	0.45	0.25
P_9	H_2N-YVAAAVVSH-NH_2	0.29	0.21

[a]BAW: Butan-1-ol:H_2O:acetic acid, 100:30:10; pH 2.6.
[b]BAWP: Butan-1-ol:H_2O:acetic acid:pyridine, 100:30:6:1; pH 4.0.

tides were purified by reversed-phase HPLC on an Ultrasphere-ODS preparative C_{18} column in 0.1% TFA with a gradient of acetonitrile. The purified peptides were homogeneous as assessed by a single peak (280 and 216 nm) on analytical RP-HPLC and a single spot by TLC (Merck Silica Gel-60 F254), except for P_9. Subsequent analysis of P_9 by quadrupole mass spectrometry (Dr. P. Alewood, Bond University) revealed the correct amino acid sequence (M_r 916).

Iodination of Synthetic Peptides With ^{125}I

Peptides P_3 to P_9, typically 20 μg of peptide in 20 μL of H_2O, were iodinated with 3–5 μL of $Na^{125}I$ (100 mCi/mL, carrier free) (Amersham) according to the procedure of Hunter and Greenwood (1962) which employed chloramine-T. In the case of tyrosine (P_1) and P_2 the reaction employed Iodobeads (Pierce) (Markwell, 1982) due to the lower incorporation of radio-iodide with chloramine-T. All iodinated products were purified by gel filtration (Sephadex-G10, 5 mL) using a disposable column (Bio-Rad).

Separation of Peptides by TLC

Peptide or assay aliquots were applied to the preadsorbent stacking area of channeled Whatman TLC plates (LK6D or LK6DF) and separated by ascending chromatography [butan-1-ol:H_2O:acetic acid, 100:30:10 (BAW), pH 2.6 or butan-1-ol:H_2O:acetic acid:pyridine, 100:30:6:1 (BAWP), pH 4.0]. Radioiodinated peptides were visualized by exposure of the TLC plate to autoradiography (Kodak XAR-5 diagnostic film) or by phosphorimaging (storage Phosphor screen, Kodak). Typically 50,000–200,000 CPM of radioactivity was spotted per lane in a 10–40 μL volume, requiring overnight exposure.

For the purposes of quantitating peptide bands the original technique required the alignment of developed autoradiograms with the TLC plates followed by band scraping. Silica fines were collected under low pressure (≈ 60 mm Hg) into a scintillation vial which was replaced after flushing the line (≈ 15 cm in length, 2 mm i.d.) with 0.5 mL of H_2O. Vials were counted in an LKB-1282 Compugamma γ-Counter (58.5% efficient) or a Minaxi Auto-Gamma Counter (5000 series) (60.8% efficient). Alternatively radioactivity was quantitated using the recently developed technology of phosphorimaging (Molecular Dynamics, CA).

Used plates, provided they had not been scraped, were regenerated for reuse by reverse (descending) chromatography using ethanol:H_2O:acetic acid (50:50:5 v/v). Regeneration of TLC plates could be performed several times (typically four times) until the plates were no longer effective in their separation of peptides. The plates were activated by prerunning in BAW or BAWP and air drying at room temperature before reuse. R_f values were found to vary significantly with these plates; however the order of the bands remained the same, and for some products separation was better (v.i.).

Assays of Peptide Hydrolysis

Intact HeLa assay. Intact HeLa cells (2 cm^2, 24-well plate, Linbro) were assayed in situ by first aspirating the conditioned media, gently washing the cells with HBSS (Hanks balanced salt solution without phenol red indicator) to remove all traces of RPMI-1640, and then incubating for up to 4 h at 37°C in 200 μL of HBSS containing approximately 3×10^5 CPM of P_9 substrate. Typically 160 μL of HBSS was first added to the cells followed by 40 μL of stock ^{125}I-P_9 containing the required radioactivity. After a fixed incubation time the assay solution was removed and 40 μL immediately applied to TLC. Inhibition studies with bestatin were performed by incubating the cells with 160 μL HBSS, containing the inhibitor at the desired concentration for 10 min, before the addition of P_9 substrate. Alternatively, the aminopeptidase inhibitor was first added to the P_9 substrate before application to the cell culture. Assaying in the presence of RPMI-1640 was found to be

inhibitory but the presence of up to 10% FBS (S70) in PBS had no effect.

Extracted HeLa assay. Butanol (5% in PBS) or Triton X-100 (0.05% in PBS) cell extracts were assayed (10 μL, 20 μL, or 40 μL aliquots) in 96-well microtitre plates in a total volume of 100 μL or 200 μL. The proteolytic assay was allowed to proceed for a specified time at 37°C.

Protease assay. Commercially obtained peptidases were assayed in a total volume of 100 μL at 37°C in which 20 μL of stock enzyme was added to 60 μL HBSS followed by 20 μL of P_9 (150,000 CPM/20 μL). Trypsin-TPCK, α-chymotrypsin-TLCK, thermolysin, dispase, HLE (human leucocytic elastase), and PPE (porcine pancreatic elastase) were each dissolved in 20 mM Tris, pH 7.6, to give a final concentration of 10 μg/mL. Leucine aminopeptidase and aminopeptidase-M, as supplied, were each diluted 1:100 with 20 mM Tris, pH 7.6, and assayed as above.

PPE was also dissolved in 100 mM Tris, pH 8.0, at 10 μg/mL from which further dilutions were made with the same buffer. Assays were performed by adding 20 μL of P_9, in HBSS, to 80 μL of different concentrations of elastase in 100 mM Tris, pH 8.0, and incubating for 30 min or 16 h at 37°C (Fig. 2). Activities hydrolyzing $Suc(Ala)_3NA$ (succinoyl trialanine para-nitroanilide), a synthetic substrate for the determination of elastase activity, were determined according to Bieth et al. (1974).

Extraction of protease activities. HeLa cells in 24-well Linbro culture plates (area of each well 2.0 cm^2) were washed free of conditioned media and non-viable cells with PBS and aspirated. The cells were then overlayed with 200 μL of 5% butanol (v/v) in PBS and allowed to stand at room temperature with occasional agitation for 5 min. The cells were released by the butanol and the contents were then transferred to an Eppendorf microfuge tube. The tissue culture well was then washed with 0.3 mL of PBS followed by a further 0.5 mL of PBS. All washes were combined with the original extract and thoroughly triturated. Alternatively all cell associated peptidase activity could be extracted with 0.1% Triton X-100 in a similar manner. The samples were then centrifuged for 5 min at 10^4 rpm (eppendorf microfuge) at room temperature. The supernatant was collected and an appropriate dilution made before assaying as described.

RESULTS

Separation of Peptides

The mobilities of the potential radio-iodinated proteolytic fragments of the nonapeptide substrate P_9 (Fig. 1) were identified by TLC. Of particular importance was the ease of separation of P_9 from all possible products, particularly

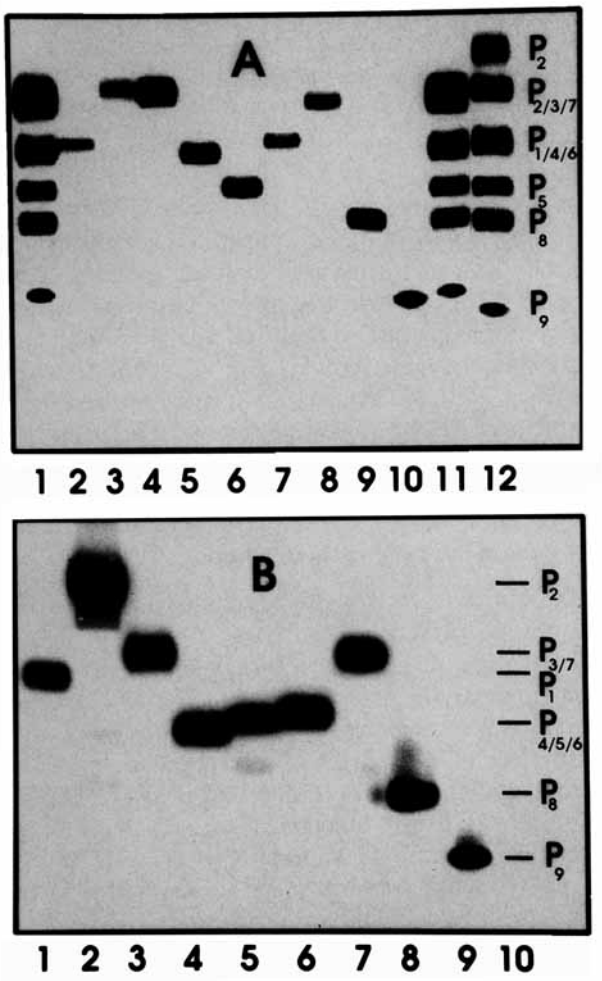

Fig. 1. Separation of radioiodinated synthetic peptides by thin layer chromatography using two different solvent systems. Peptides were visualized by autoradiography with Kodak XAR-5 film and are presented as densitometer images. **A:** Lanes 1 and 11 are a mixture of all peptides (P_1–P_9 inclusive) and lanes 2–10 are P_1 to P_9 individually (cf. Table I). Lane 12 is a mixture of all peptides separated on a TLC plate prerun in BAW and air dried prior to sample application. **B:** Lanes 1–9 show P_1–P_9 respectively; lane 10 shows the positions of P_2, $P_{1/3/7}$, $P_{4/5/6}$, P_8, and P_9, respectively. Layer: Whatman LK6D(F) Silica Gel G60A; Solvent (A): Butan-1-ol, H_2O, acetic acid (100:30:10; BAW); (B): butan-1-ol, H_2O, acetic acid, pyridine (100:30:6:1; BAWP); Chromatography: 5 h.

P_5, which would be the expected product if the cleavage site observed in proTGF$_\alpha$ was recognized within the P_9 substrate and was hydrolyzed by the appropriate protease (cf. Discussion). Adequate separation between P_2, P_3, and P_7 as well as P_1, P_4, and P_6 was not achieved using the BAW solvent system (Fig. 1A). Prerunning the TLC plate in BAW before application of the peptides, however, did result in the separation of P_2 from $P_{3/7}$ (Fig. 1A, lane 12) as did changing the ratio of solvents from 100:10:30 to 100:30:10 (as seen in Fig. 6). BAWP (Fig. 1B) allowed the distinct separation of P_1 from $P_{4/6}$. P_2, P_8, and P_9 were distinctly separated by BAWP but $P_{3/7}$ were not resolved nor were $P_{4/6}$ which now ran with P_5, although P_5 was distinct in BAW. The mobilities of P_1 and P_5 were both found to be sensitive to slight changes in pH when BAWP was used. No solvent system was found to resolve $P_{3/7}$ and $P_{4/6}$ satisfactorily.

Radioiodination of P_9

A typical iodination of 20 μg of P_9 resulted in 10^8 CPM (1.71×10^8 DPM) of labelled peptide collected in a total volume of 1 mL. The specific activity of the peptide (corrected for decay) was calculated to be 3.85 μCi/μg (7.84×10^6 CPM/nmol) based on an M_r of 916 for P_9 and an assumed recovery of 100% by gel filtration. This compares with a theoretical maximum of 2400 μCi/μg (^{125}I, 2200 Ci/mAtom) based on 100% isotopic abundance and the incorporation of 1 mol of ^{125}I per mol of P_9. It follows that the concentration of ^{125}I-P_9 employed in the assays was 0.5 nM with the concentration of the unlabelled P_9 being no greater than 200 nM which must be considered an overestimate. These concentrations are much lower than typical K_m values in the range of 10 μM–1 mM for synthetic peptide substrates with HLE and PPE (Castillo et al., 1979; Del Mar et al., 1980), which suggests that the concentration of P_9, labelled or not, will be much lower than the K_m for the peptidase(s) which will act on it. Under these conditions the initial reaction velocity of the assay should be proportional to the substrate concentration and thus be first order with respect to substrate (v.i.).

Hydrolysis of P_9 by Commercially Prepared Enzymes

Several proteases were examined for their activity towards P_9 (Fig. 2). Leucine aminopeptidase and aminopeptidase-M both yielded P_1 whereas the neutral metalloendopeptidase thermolysin exhibited broad specificity in hydrolyzing P_9 to generate P_1, $P_{3/7}$, and $P_{5/6}$. Dispase produced $P_{5/6}$ and P_4 although a trace of P_1 and $P_{3/7}$ could also be detected upon overexposure of the radioimage. TPCK-treated trypsin and TLCK-treated α-chymotrypsin showed no activity towards P_9. PPE and HLE hydrolyzed P_9 to yield P_2 and P_3 with P_4 and P_5 also being detected with PPE but not with HLE. P_3 was identified as a major product as the peptides P_6 to P_9 gave the same proteolytic product thus eliminating P_7 as a confounder (results not presented). Intriguingly PPE at 25 ng/mL was unable to hydrolyze P_5 as demonstrated in Figure 3 (cf. lanes 17, 18). In fact, the proportion of P_2:P_3:P_4:P_5 formed was found to be the same regardless of the concentration of PPE employed and the time of incubation or pH of the assay.

In a separate experiment the concentration of PPE activity was standardized from a series of assays done in triplicate with the substrate

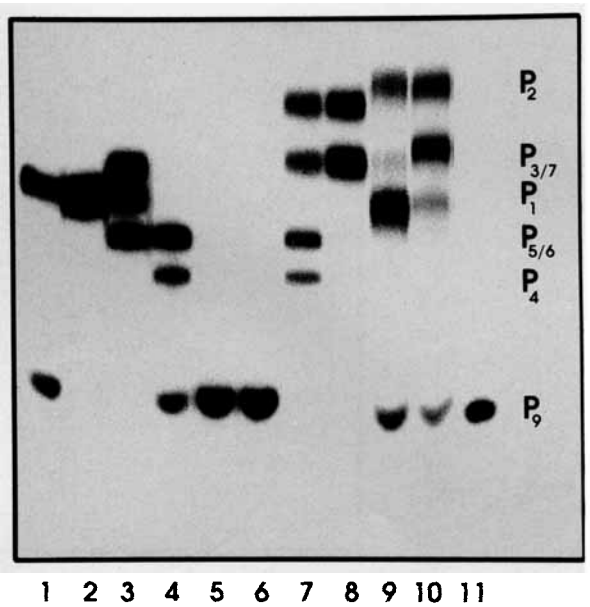

Fig. 2. Densitometer image of an autoradiograph showing the susceptibility of P_9 to hydrolysis by peptidases. Time of assay was 30 min for commercial proteases and 120 min for confluent cultures of intact cells. Solvent system was BAWP and the TLC plates were run for 5 h. The peptidases are (by lane) leucine aminopeptidase (1); aminopeptidase M (2); thermolysin (3); dispase (4); trypsin-TPCK (5); α-chymotrypsin-TLCK (6); PPE (7); and HLE (8). Also shown are the products of assay media from intact HeLa cell cultures preincubated without (9) or with (10) bestatin before the addition of P_9. The P_9 substrate standard (11), in HBSS, was preincubated at 37°C for 96 h (sterile).

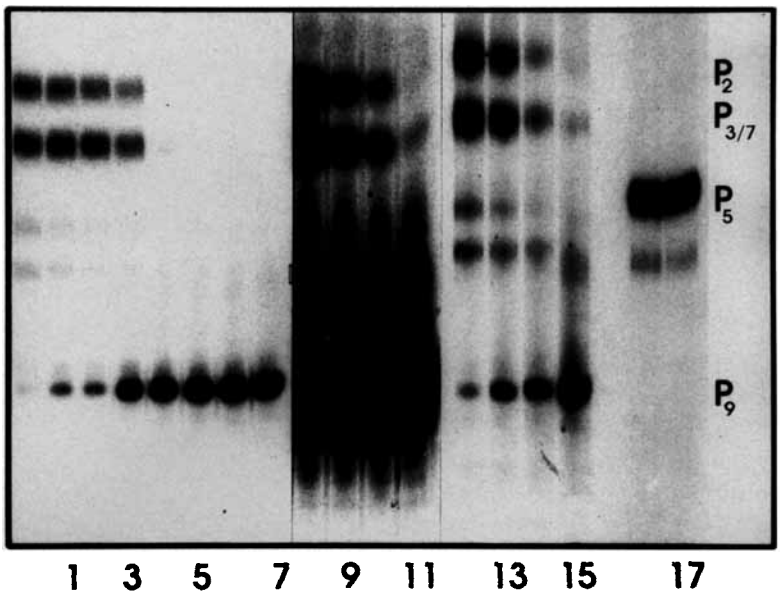

Fig. 3. Sensitivity of the assay to various concentrations of PPE. Lanes 1–8 represent a 30 min incubation of P_9 at 37°C with PPE at 200, 100, 50, 25, 5, 2.5, 1, and 0.5 ng/mL respectively. Lanes 9–12 are the same as lanes 5–8 but are overexposed to autoradiography. Lanes 13–16 represent a 16 h incubation of P_9 at 37°C with PPE at 5.0, 2.5, 1.0, and 0.5 ng/mL. Lane 17 is the P_5 control which has a slower migrating contaminant. Lane 18 shows P_5 incubated with PPE at 50 ng/mL for 30 min. Solvent system employed was BAWP and the autoradiographs are presented as densitometer images.

$Suc(Ala)_3NA$ according to the procedure of Bieth et al. (1974). PPE at 10–100 ng/mL typically hydrolyzed 10–80% of the substrate P_9 at 37°C after a 30 min incubation (Fig. 3). Overexposure of autoradiograms has further indicated that concentrations of PPE at 0.5 ng/mL resulted in distinct hydrolysis of P_9 after only a 30 min incubation and effected $\approx$20% hydrolysis of P_9 after 16 h of sterile incubations (Fig. 3, lanes 13–16). Control, sterile assays at 37°C resulted in no autohydrolysis of P_9 after 96 h (Fig. 2, lane 11). In contrast, with $Suc(Ala)_3NA$ as substrate at 1 mM, a 4 h incubation did not reliably detect activity with 25 ng/mL of PPE.

Subculturing in Peptidase-Free Media

It is important to note that control assays with fresh media (S56) alone also revealed significant proteolytic activity towards P_9 which was shown to be present in FBS. P_1 was the predominant product, attributable to aminopeptidase activity, although bestatin-insensitive proteases were also detected (results not presented). The removal of serum proteases was essential in that their presence could obscure the interpretation of the origin of cell-associated activity. Heat inactivation of FBS at 69–70°C for 1 h (S70) was necessary for the complete removal of proteolytic activity against P_9, after which the serum was still able to support cell growth. Heat treatment at 72°C resulted in the serum becoming too viscous to filter and unable to support cell culture when added to RPMI-1640.

HeLaN70 cells were established by subculturing HeLaN56 once in S70 media before seeding in 24-well plates for assay. Many cells adapted poorly to the 70°C inactivated serum as evidenced by clumped colonial growth and poor adherence to the culture flasks. The first few passages in S70 eliminated these poorly adherent cells and allowed for the loss of contaminating protease activity that may have adsorbed to the cells during prior growth in S56 medium. Cells that readily adapted formed colonies that were looser in form without the close cell-cell apposition characteristic of the parent HeLaN56, but preserved a similar growth rate to that in S56 medium. Consequently cell cultures appeared to reach confluency much quicker in S70 medium than did their counterparts in S56 medium.

Cell Associated Peptidase Activity

Intact HeLa cells, cultured in S70 media, hydrolyzed P_9 (Figs. 2, 6) with the appearance of P_1 as the major product band, indicative of ami-

nopeptidase activity. This was verified by the addition of 10 μM bestatin (an inhibitor of aminopeptidases, Suda et al., 1976) to the assay, which eliminated the formation of P_1 and resulted in P_3 and P_2 as the major products. The effect of bestatin was similar, with or without a 15 min preincubation with the cells, prior to assay. The R_f of P_2 was increased slightly relative to adjacent lanes (Fig. 2) as is usually observed when the substrate has been incubated with intact cell cultures. That the band was P_1 was further verified by separation of peptides with BAWP and by performing a series of assays with monolayers of intact cells using the various peptides in the absence of bestatin. The final product band was P_1 as evidenced by an assay with P_3 as substrate (results not presented). P_3 was hydrolyzed to a band which ran with $P_{1/4/6}$ and could therefore only be P_1. Furthermore this same product band was generated by incubating P_9 with commercially obtained aminopeptidases (Fig. 2). Interestingly, incubation of HeLa cells with the various peptides (results not presented) revealed that for smaller peptides (P_4–P_7) the product P_2 was relatively more abundant than that following the hydrolysis of P_8 and P_9.

Peptidase Extraction

Peptidases responsible for the proteolytic degradation of P_9 could be effectively extracted (>95%) with 5% butanol in PBS or completely with 0.05% Triton X-100 as determined from assays with the extracts and resulting cell pellet. Peptidase activity was not extracted by a 5 min incubation with butanol up to 2% in which cell viability was retained as determined by the absence of trypan blue staining and the ability of cells to resume replication. Extraction with 3–4% butanol resulted in less than 10% of the activity being removed but with the cells becoming increasingly non-viable. At >7% butanol the overall activity of the extracted peptidases decreased relative to 5% butanol extraction, a decrease which was not reversible upon dilution of the butanol. Peptidase activity was, therefore, presumed to be inactivated by concentrations of butanol >7%.

Five percent butanol caused immediate "blebbing" of the surface of the cells (Jewell et al., 1982; Lemasters et al., 1987) which readily detached within minutes and stained with trypan blue. The use of 0.05% Triton X-100 in PBS caused complete and immediate lysis with dissolution of the cell membrane.

Linearity of Peptidase Activity

While it will become clear that several enzymes capable of cleaving P_9 are present in butanol extracts of HeLa cell cultures, it was useful to know whether the total activities monitored conformed to standard enzyme kinetics so that differences in such activity could be quantified. Therefore, the peptidase activity of HeLa cell extracts as well as that of intact cell cultures was examined as a function of cell extract, volume, and time.

With Cell Extracts

Culture plates were seeded in duplicate with varying numbers of cells and incubated for 3 days. The cultures were then extracted with butanol and cell numbers counted using aliquots of the triturated suspension before centrifugation of the extract. Figure 4A shows that despite the low substrate concentration (<0.2 μM) there was a linear relationship between the percent hydrolysis of P_9 in a standard time '30 min' and enzyme activity causing ≤50% conversion of the substrate. The three curves illustrated define three sets of data generated by adding 10 μL, 20 μL, and 40 μL of extract from each culture to the fixed volume assay. Linear regression using only those assays with less than 50% hydrolysis yielded excellent correlation coefficients (r = 0.9901, 0.9856, and 0.9566 respectively). The ratios of the slopes of the lines were 4.29 (± 0.55, the 95% confidence interval): 2.00 (± 0.19): 1.23 (± 0.43) which are in good agreement with the ratio of the volume of the extract assayed (4:2:1), so that the rate of the reaction is, to a useful approximation, proportional to the enzyme concentration up to 50% substrate hydrolysis.

Since the low substrate concentrations used in these assays would be expected to be nonsaturating for the protease activities, we would anticipate first-order kinetics to prevail, given that the hydrolyses are linear with time (v.i.). We have therefore plotted the data of Figure 4A as $-ln$ (concentration of unhydrolyzed P_9 divided by the initial concentration of P_9 measured as the sum of all bands) $[-ln(P_9/\Sigma P_i)]$ vs. cell density (standardized as cell density equivalents of the added extract) to see if this function was linear. Although the data of Figure 4B are well

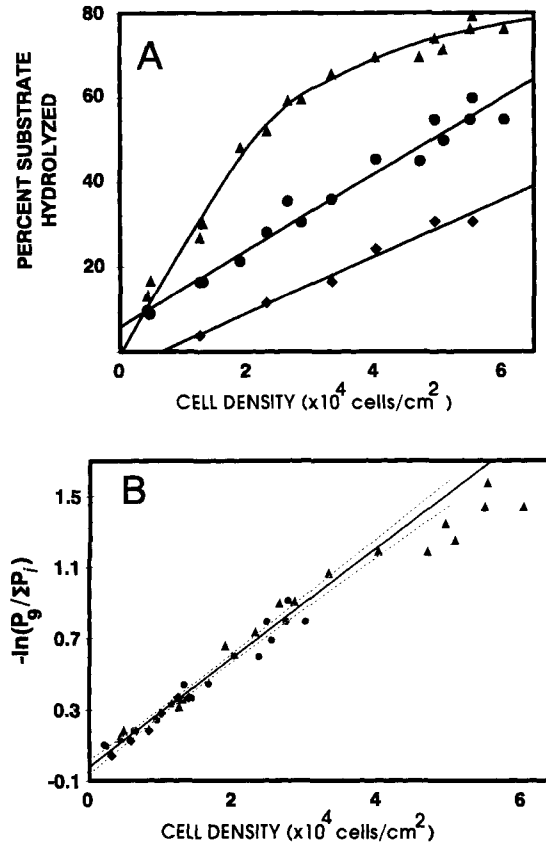

Fig. 4. Characterization of the extracted HeLa cell assay. **A:** Quantitative analysis of TLC for the percent hydrolysis of substrate (P_9) with 10 μL (◆), 20 μL (●), and 40 μL (▲) aliquots of HeLa cell extracts from cultures at various densities. $P_9/\Sigma P_i$ represents the amount of radioactivity under the P_9 band, divided by the sum of all radioactivity recovered from TLC scrapings [$CPM_{total} = (1.7 \pm 0.06) \times 10^5$, SEM]. Cell extracts were assayed in a total volume of 100 μL for 1 h. TLC solvent was BAW and ran for 3.5 h. **B:** Represents the logarithmic transformation of the above results in which the 10 μL or 20 μL assays have been adjusted to represent a 40 μL assay at ¼ or ½ the cell density respectively. The data for all values corresponding to less than 70% hydrolysis were used to calculate the regression, yielding a correlation coefficient $r = 0.9844$, while the 95% confidence limits for the estimates of $-ln(P_9/\Sigma P_i)$ are shown as dotted lines.

described by linear regression ($r = 0.985$) for all values of substrate hydrolysis (< 80%) it is apparent that the set of data greater than 70% hydrolysis [$-ln(P_9/\Sigma P_i) > 1.2$] lies outside the 95% confidence interval about the regression line on the rest of the data. The six values which fall below the regression line (all 40 μL of extract) deviate from first-order kinetics at these extreme values of hydrolysis because the values of hydrolytic activity obtained with lesser amounts of the same extracts (10μL and 20 μL vs. 40 μL for cell densities > 4.5×10^4 /cm^2) did in fact fall on the regression line.

The enzyme assay proved to be linear with time. Figure 5A shows representative data for the percent hydrolysis of substrate with increasing time of incubation in the absence of bestatin. Again 10 μL, 20 μL, or 40 μL volumes of cell extract were employed and resulted in linear plots for all three assays ($r = 0.999$, 0.999, and 0.996) where the degree of hydrolysis was less than 50%. The ratios of the slopes were in agreement with those of the volume of extract assayed [4.49 (± 1.53):2.00 (± 0.13):1.14 (± 0.10)].

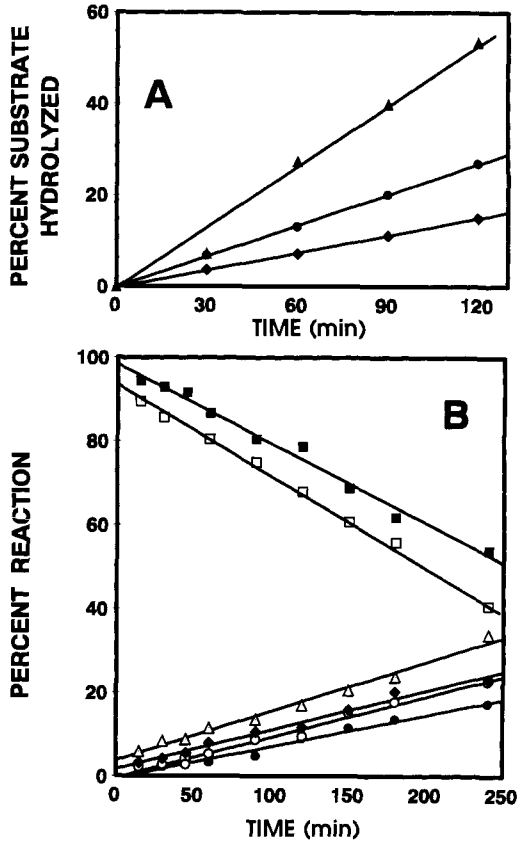

Fig. 5. Linearity of substrate hydrolysis as a function of incubation time. **A:** Extracted cells; 10 μL (◆), 20 μL (●), and 40 μL (▲) aliquots from a 5% butanol extract of intact cells at a density of 2.3×10^4 cells/cm^2 (cf. Fig. 4) were assayed for 30, 60, 90, and 120 min in the absence of bestatin. **B:** Intact cells; a series of 24-well plates, seeded at 15×10^4 cells/cm^2 and left to incubate for 48 h until they reached confluency (24×10^4 cells/cm^2). Each point represents a separate culture. Iodinated species monitored were P_9 (□), P_1 (△), P_2 (○); the filled symbols, (■), P_9; (●), P_2; (◆), P_3 respectively, resulted from assays performed in the presence of bestatin. In both experiments the % hydrolysis was determined by scraping the TLC plate and counting the ^{125}I in a γ-counter.

These experiments show for cell extracts over the cell densities investigated, that there was useful linearity between cell number, volume of extract assayed, incubation time, and the percent P_9 hydrolyzed for values $< 50\%$.

With Intact Cells

The effect of cell density on peptidase activity, associated with intact HeLa cultures, was also investigated. Figures 6A and B are representative radioimages showing the increased levels of P_9 hydrolysis with increasing cell density in the presence (Fig. 6A) or absence (Fig. 6B) of bestatin. The proportion of P_9 not yet hydrolyzed was quantitated from duplicate assays, performed on separate cultures, and normalized to a cell density of 25×10^4 cells/cm^2 (the density corresponding to 100% confluence) to determine whether the degree of hydrolysis varied with cell density on a per cell basis (Fig. 6E). Within the error of the experiment, the resulting curves were linear with zero slope indicating a constant level of total peptidase activity on a per cell basis for intact cultures varying from 40% confluence to a tightly packed monolayer.

Cultures assayed in the presence or absence of bestatin exhibited linearity for the percent hydrolysis of P_9 ($r > 0.980$) with time (Fig. 5B). Similarly the kinetics of appearance of product bands (P_1, P_3) were also linear and the experimental curves were described by linear regression with correlation coefficients better than 0.940. The appearance of P_2, however, had a lag period of 50–60 min which implies more complex steps leading to its appearance. For this reason quantitation of experiments addressing total activity were simply equated with percent hydrolysis of P_9 rather than the percent production of P_i ($i = 1$–8).

For comparison, the assays for total cell extracts of this experiment are included in Figure 6. The radioimaged results are presented alternatively as a 3D-projection. Quantitation of the results showed that a 125-fold dilution of the original 1 mL extract, for a particular cell culture, was required in the final assay to result in a level of hydrolysis comparable to that observed for the intact cell assay (Fig. 6).

DISCUSSION

Several well defined enzymes recognize and hydrolyze P_9 (YVAAAVVSH) generating a number of peptide products. The suitability of P_9 as a substrate for thermolysin-like enzymes as well as the elastases was evident and variations in the specificities of these enzymes were apparent. Dispase has been described as similar to thermolysin in substrate specificity and may be used in place of thermolysin in protein sequencing studies. However, a notable difference in our assay was that dispase generated P_5 from P_9 whereas thermolysin did not. PPE and HLE both cleaved P_9 to generate P_2 and P_3 as major products thereby showing no obvious preference for an alanine over a valine or vice versa. However PPE also generated P_4 and P_5 (YVAAA), concurring with prior observations that PPE has a preference to cleave C-side to Ala (Werb et al., 1982). To define the nature of the cleavage pattern, an independent assay in which P_5 and P_8 were added as substrates for PPE resulted only in P_8 generating P_2 and P_3 whereas there was no hydrolysis observed for P_5. These data emphasize the importance of an extended sequence about the scissile bond to confer specificity for the elastase group of enzymes (Bode et al., 1989).

The tendency for the products P_2 and P_3 as opposed to P_5 to be formed from P_9 by both elastases, which have been shown to release bioactive TGF_α from its precursor (Mueller et al., 1990; Ignotz et al., 1986), and by cell surface endopeptidases (this study) may be explained by considering subsite specificities. In a larger polypeptide such as preproTGF$_\alpha$, the reported specificity of cleavage may depend on distal subsite interactions and structural factors. Alternatively, 'wobble' in the site of cleavage of preproTGF$_\alpha$ may actually occur (Wong et al., 1989), such that hydrolysis of $V_{36}\dagger A_{37}$ or $A_{37}\dagger A_{38}$ in preproTGF$_\alpha$, equivalent to generating P_2 and P_3 from P_9, would still result in the release of bioactive TGF_α. N-terminal sequencing of TGF_α from a variety of different natural sources has not yet been done to verify the uniqueness of $A_{39}\dagger V_{40}$ or $A_{89}\dagger V_{90}$ cleavage nor have the scissile bonds resulting from experimental elastase excision of TGF_α from its precursor been identified. Most interesting is the observation that similar enzymes, in this case the elastases, can produce different product patterns which are recognized by characteristic proportions of radioiodinated cleaved peptides. Such cleavage profiles may prove useful in recognizing individual enzyme specificities as well as indicating preferential scissile bonds for refining structure-function studies. It will be of considerable interest to

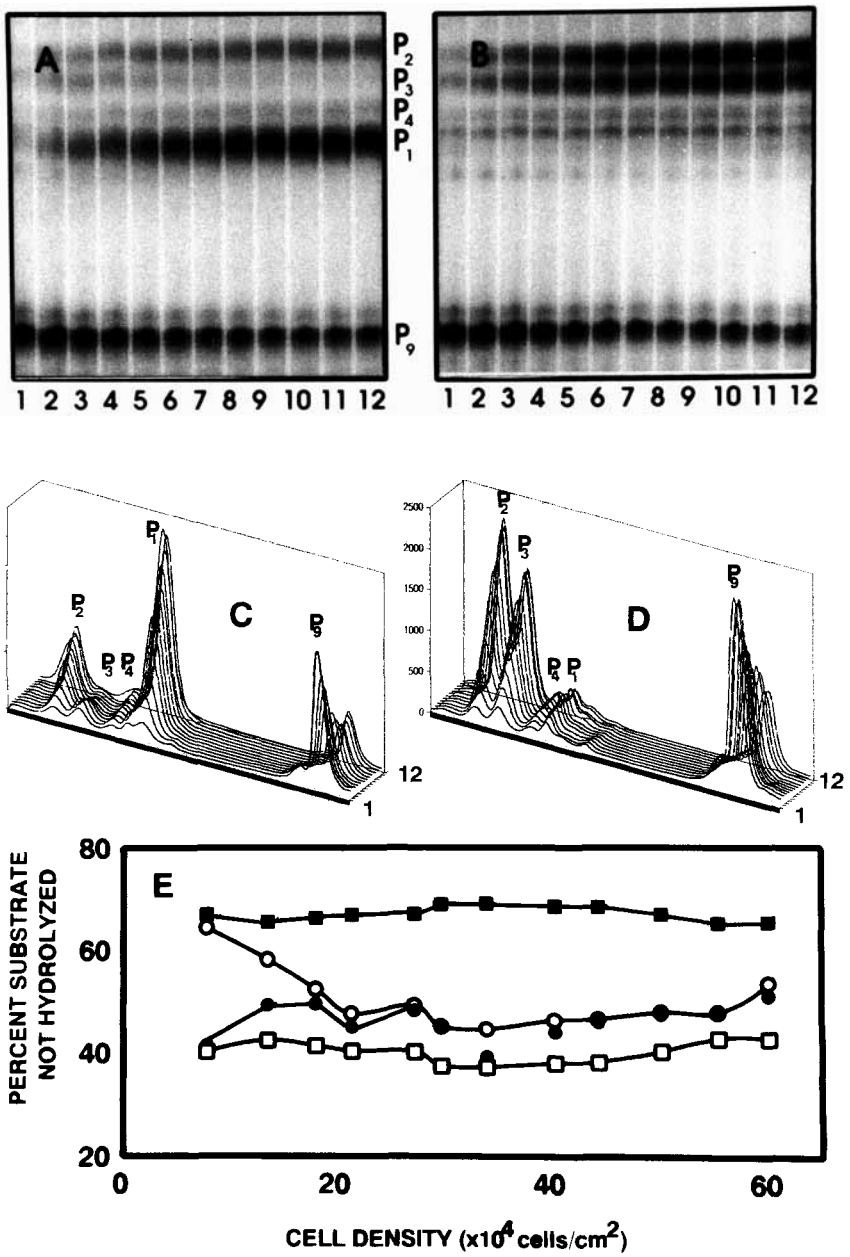

Fig. 6. Effect of cell density on the linearity of the intact and extracted HeLa cell assay. Intact HeLa cultures at 7.8, 13.5, 18.0, 21.4, 27.3, 29.8, 34.0, 40.4, 44.4, 50.5, 55.5, and 60.1 × 10^4 cells/cm^2 (lanes 1–12) were assayed in 24 well-plates (2 cm^2) for 2 h at 37°C. Assay media were removed and applied to TLC plates and products separated using a modified BAW solvent system (100:10:30, butanol:H_2O:acetic acid). The developed thin layer chromatograms were phosphorimaged and representative radioimages are presented for assays in the absence (**A**) or presence (**B**) of bestatin. Intact cultures were also extracted with 5% butanol and diluted 125-fold with PBS and assayed in the absence (**C**) or presence (**D**) of bestatin for 2 h in a total volume of 200 μL. Results for (C) and (D) are presented as 3D-contour maps for the extracted cell assays in which the width of each lane was reduced to a mean pixel value over the length of the lane (114 pixel values per cm of scanned image). In (**E**) the data of A (○), B (●), C (□), and D (■) and their duplicate assays, performed on separate cultures at the specified densities, were quantitated with a phosphorimager and are presented as the proportion of P_9, not yet hydrolyzed, which has been normalized to a cell density of 25 × 10^4 cells/cm^2. Cell densities were determined from the mean cell count of a minimum of eight cultures at each density.

determine the effects of extending the substrate currently used to determine the effects of other structural features of the TGF_α molecule on the specificity of defined proteases for the presumed A†VV scissile bond.

A series of experiments to determine the sensitivity of the assay revealed that PPE at concentrations as low as 25 ng/mL was detectable after 30 min with greater than 50% turnover of P_9. Overexposure of the autoradiograms revealed that PPE at 0.5 ng/mL was detectable in the hydrolysis of P_9 although resulting in $< 1\%$ turnover of substrate. Allowing the incubation period to increase to 16 h resulted in $\approx 20\%$ hydrolysis. This compares with a limit of detectability of 50 ng/mL for PPE with $Suc(Ala)_3NA$ as substrate (Bieth et al., 1974; Castillo et al., 1979). Detection limits of 0.15 ng/mL with benzylthiol peptides and 0.47 ng/mL with aminomethylcoumarins for PPE have been reported (Castillo et al., 1979).

This method of assay also facilitates microadaptation. We are currently estimating proteolytic activity on surface epithelia in an area of 0.03 cm^2 ($\approx 5{,}000$ cells) in 5 μl of assay mix. Since proportionate hydrolysis ($P_9/\Sigma P_i$) is, to a close approximation, linear with enzyme hydrolysis ($\leq 50\%$), quantitative recovery of the assay fluid is unnecessary making the manipulations simple. We have also applied the assay successfully to the detection of thermolysin which was separated by capillary electrophoresis from a single run with nanolitre sampling. The employment of radioiodinated peptides as substrates has been previously reported. Kenny and coworkers (Kenny, 1977; Fulcher and Kenny, 1983) used ^{125}I-insulin B chain for probing cell surface protease activity which initially revealed the existence of endopeptidase-24.11. More recent work by Vanneste et al. (1990) has examined the metabolism of brain natriuretic peptide in vivo. In vitro assays with endopeptidase-24.11 (1 μg/mL) and angiotensin-converting enzyme (20 μg/mL) resulted in hydrolysis of their natural substrate at a concentration of 1.4 nM (V = 200 μL). Interestingly this group also reported the ability of serum to effect hydrolysis of the natriuretic peptide. Similarly Nyberg et al. (1990) have used a radioiodinated nonapeptide to purify and characterize an aminopeptidase from human cerebrospinal fluid where they employed μM concentrations of substrate. In all cases HPLC was the method used for the quantitation and analysis of results. We have found that TLC has provided a simple, cost-effective, rapid method for analysis of peptidase product while maintaining sensitivity allowing the detection of sub *f*mol levels of product, assayed in μL volumes of assay mixture.

Intact monolayer cultures of HeLa cells, covering less than 2 cm^2, were active in the hydrolysis of P_9 after exogenous proteolytic activity was removed from the growth media. The peptidases responsible for hydrolysis are associated with the cell since neither conditioned media nor salt and mild detergent washes of intact cells showed activity towards the substrate. These findings strongly suggest that the peptidase(s) of interest are membrane associated but does not rule out the possibility of protease secretion during the course of the assay. Amino(exo-) peptidase activity dominated in assays of intact cells. Umezawa and coworkers (Umezawa and Aoyagi, 1977) found unexpectedly high exopeptidase activity compared with endopeptidase activity on the surface of mammalian cells. Many exopeptidases have also been partially characterized and have been shown to be associated with plasma membranes (Kenny, 1977) and constitute members of the ectoenzyme family (Kenny et al., 1987). In the absence of specific inhibitors (e.g., bestatin) these exopeptidases mask the endopeptidase activity because they can readily hydrolyze the smaller peptide products of P_9 (P_3–P_8).

Aminopeptidase activity was easily measured and eliminated in the peptide assay. Assays performed in the presence of bestatin (a general inhibitor of aminopeptidases) resulted in anywhere from 0–20% less proteolytic activity towards P_9 in different experiments, with the major product P_1 being replaced by P_3, while P_2 remained relatively unchanged. Typically, when the same number of intact cells were extracted, a 100-fold dilution of the extract in the final assay was required for a comparable percent hydrolysis of substrate as was observed for intact cells. Thus, the putative cell surface peptidase activity is only about 1% of the total cell activity for both amino- and non-amino peptidase activities. Interestingly, ectopeptidases and other ectoenzymes [specifically aminopeptidases (Louvard, 1980)] circulate via intracellular routes of membrane traffic (Luzio et al., 1987).

The hydrolysis of P_9 by intact or extracted cells was kinetically uncomplicated and most easily performed under limiting substrate condi-

tions. Our results suggest that with increasing cell density the level of cell-associated peptidase activity for intact or extracted cultures was constant.

We have thus established the suitability and applicability of a nonapeptide substrate cognate with the cleaved amino acid sequence of TGF_α to monitor cell-associated proteases. The assay is highly sensitive and has provided a useful proteolytic probe for the identification of enzyme(s) that will specifically hydrolyze it. These results have demonstrated the predominance of exopeptidase activity but with appropriate inhibitors or peptide modifications, non-aminopeptidases (presumably endopeptidases) can be distinguished. Purification and kinetic characterization of candidate $proTGF_\alpha$-ases is underway.

ACKNOWLEDGMENTS

This work was supported by the National Health and Medical Research Council of Australia and the Queensland Cancer Fund.

REFERENCES

Atlas D, Berger A (1972): On the specificity of elastase. Hydrolysis of peptide p-nitrobenzyl esters. Biochemistry. 11:4719–4723.

Bieth J, Speiss B, Wermuth CG (1974): The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. Biochemical Medicine 11:350–357.

Bode W, Meyer E, Jr, Powers JC (1989): Human leukocyte and porcine pancreatic elastase: X-ray crystal structures, mechanism, substrate specificity, and mechanism-based inhibitors. Biochemistry 28:1951–1963.

Brachmann R, Lindquist PB, Nagashima M, Kohr W, Lipari T, Napier M, Derynck R (1989): Transmembrane TGF-α precursors activate EGF/TGF-α receptors. Cell 56:691–700.

Castillo MJ, Nakajima K, Zimmerman M, Powers JC (1979): Sensitive substrates for human leukocyte and porcine pancreatic elastase: A study of the merits of various chromophoric and fluorogenic leaving groups in assays for serine proteases. Anal Biochem 99:53–64.

Chen TR (1977): In situ detection of Mycoplasma contamination in cell cultures by fluorescent Hoechst 33258stain. Exp Cell Res 104:255–262.

Del Mar EG, Largman C, Brodrik JW, Fassett M, Geokas MC (1980): Substrate specificity of human pancreatic elastase 2. Biochemistry 19:468–472.

Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV (1984): Human transforming growth factor-α: Precursor structure and expression in E. coli. Cell 38:287–297.

Ellem KAO, Cullinan M, Baumann KC, Dunstan A (1988): UVR induction of TGF_α: A possible autocrine mechanism for the epidermal melanocytic response and for promotion of epidermal carcinogenesis. Carcinogenesis 9:797–801.

Fulcher IS, Kenny AJ (1983): Proteins of the kidney microvillar membrane. The amphipathic forms of endopeptidase purified from pig kidneys. Biochem J 211:743–753.

Geneste P, Bender ML (1969): Esterolytic activity of elastase. PNAS (USA) 64:683–685.

Houghten RA (1985): General method for the rapid solid phase synthesis of large numbers of peptides: Specificity of antigen-antibody interaction at the level of individual amino acids. PNAS (USA) 82:5131–5135.

Hunter WM, Greenwood FC (1962): Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature 194:495–496.

Ignotz RA, Kelly B, Davis RJ, Massague J (1986): Biologically active precursor for transforming growth factor type α, released by retrovirally transformed cells. PNAS (USA) 83:6307–6311.

Jewell SA, Bellomo G, Thor H, Orrenius S, Smith MT (1982): Bleb formation in hepatocytes during drug metabolism is caused by disturbances in thiol and calcium ion homeostasis. Science 217:1257–1259.

Kenny AJ (1977): In Barrett AJ (ed): "Proteinases in Mammalian Cells and Tissues." Amsterdam: Elsevier/North Holland Biomedical Press, pp 393–444.

Kenny AJ, Stephenson SL, Turner AJ (1987): Cell surface peptidases. In Kenny AJ, Turner AJ (eds): "Mammalian Ectoenzymes." Amsterdam: Elsevier/North Holland Biomedical Press, pp 169–210.

Knight CG (1977): Principles of the design and use of synthetic substrates and inhibitors for tissue proteinases. In Barrett AJ (ed): "Proteinases in Mammalian cells and Tissues." Amsterdam: Elsevier/North Holland Biomedical Press, pp 583–636.

Lee DC, Rose TM, Webb NR, Todaro GJ (1985): Cloning and sequencing analysis of a cDNA for rat transforming growth factor-α. Nature 313:489–491.

Lemasters JJ, DiGuiseppi J, Nieminen AL, Herman B (1987): Blebbing, free Ca^+ and mitochondrial membrane potential preceeding cell death in hepatocytes. Nature 325:78–81.

Louvard D (1980): Apical membrane aminopeptidase appears at site of cell-cell contact in cultured kidney epithelial cells. PNAS (USA) 77:4132–4136.

Luzio JP, Barron MD, Bailyes EM (1987): Cell Biology. In Kenny AJ, Turner AJ (eds): "Mammalian ectoenzymes." Amsterdam: Elsevier/North Holland Biomedical Press, pp 111–138.

Markwell MAK (1982): A new solid-state reagent to iodinate proteins. Anal Biochem 125:427–432.

Moore ML, Bryan WM, Fakhoury SA, Magaard VW, Huffman WF, Dayton BD, Meek TD, Hyland L, Dreyer GB, Metcalf BW, Strickler JE, Gorniak JG, Debouck C (1989): Peptide substrates and inhibitors of HIV-1 protease. BBRC 159: 420–425.

Mueller SG, Paterson AJ, Kudlow JE (1990): Transforming growth factor α in arterioles: Cell surface processing of its precursor by elastases. Mol Cell Biol 10:4596–4602.

Nyberg F, Thornwall M, Hetta J (1990): Aminopeptidase in human CSF degrades delta-sleep inducing peptide (DSIP). BBRC 167:1256–1262.

Powers JC, Harper JW (1986): Inhibitors of serine proteases. In Barrett AJ, Salveson G (eds): "Proteinase Inhibitors. Research Monographs in Cell and Tissue Physiology," Vol 12. Amsterdam: Elsevier/North Holland, pp 54–152.

Stein RL, Strimpler AM (1987): Catalysis by human leukocyte elastase: Proton inventory as a mechanistic probe. Biochemistry 26:1305–1314.

Suda H, Aoyagi T, Takeuchi T, Umezawa H (1976): Inhibition of aminopeptidase-B and leucine aminopeptidase by bestatin and its stereoisomer. Arch Biochem Biophys 177: 196–200.

Umezawa H, Aoyagi T (1977): Activities of proteinase inhibitors of microbial origin. In Barrett AJ (ed): "Proteinases in Mammalian Cells and Tissues." Amsterdam: North Holland Publishing Co., pp 637–662.

Vanneste Y, Paulwels S, Lambotte L, Deschodt-Lanckman M (1990): In vivo metabolism of brain natriuretic peptide in the rat involves endopeptidase 24.11 and angiotensin converting enzyme. BBRC 173:265–271.

Werb Z, Banda MJ, McKerrow JH, Sandhaus RA (1982): Elastases and elastin degradation. J Invest Dermatol 79: 154s–159s.

Wong ST, Windell LF, McCune BK, Earp HS, Teixido J, Massague J, Herman B, Lee DC (1989): The TGF_{α} precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction. Cell 56:495–506.