Protease effects on specific growth properties of normal and transformed baby hamster kidney cells

(anchorage dependence/serum requirement/saturation density/plasmin/trypsin active-site inhibitors)

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ABSTRACT   We have tested the effects of exogenous pro-
teases on the growth of normal and transformed hamster fi-
broblasts in the classic culture assays for transformation. The
results indicate that exogenous proteases act to decrease the
serum requirement of normal cells but not nearly to the extent
that occurs in the process of viral transformation. Proteases do
not further decrease the serum requirement of transformed cells,
nor do they affect the maximal saturation density or the plating
efficiency in soft agar of either normal or transformed cells.
Under conditions optimal for growth stimulation, proteases
decrease the strength of cell-to-substrate adhesion but do not
affect cellular morphology. In contrast to previous studies, ex-
periments using highly purified trypsin and several different
active-site inhibitors strongly suggest that the growth-stimula-
tory activity of trypsin is not directly related to the proteolytic
activity of the molecule.

Many lines of evidence suggest that proteolytic enzymes may
play important roles in malignancy and in cellular transfor-
mation in vitro (1–3). In particular, the cellular production of
an activator of the serum zymogen plasminogen has been im-
plicated as a specific correlate of malignant transformation
(4–11). In turn, the activated protease plasmin is reported to
determine important aspects of the phenotype of transformed
cells, including the ability to multiply upon suspension in
semisolid medium (5, 8, 12, 13). This latter characteristic may
be the in vitro criterion most accurately diagnostic of the on-
cogenic potential of a cell in vivo (8, 9, 14–22). To the contrary,
other studies suggest that none of the standard in vitro criteria
for transformation are reliable indicators of tumorigenicity
(23–29). In many cell types, a simple relationship between
growth in semisolid medium and plasminogen activator is not
apparent (22, 30–32). Furthermore, although high levels of
plasminogen activator are usually found in human and mouse
sarcomas (33–35), high concentrations are also observed in
various normal tissues (7, 33–43). In contrast, carcinomas in
these two species are generally found to be deficient in plas-
minogen activator (33, 35, 36, 44, 45). Therefore, the extent of
protease involvement in malignancy and in cellular transfor-
mation is still uncertain.

One approach to elucidating the potential roles of proteases
in cellular transformation has been to analyze the effects of
purified proteases on cells in vitro (46, 47). Normal fibroblasts
have been exposed to a wide variety of proteases and the results
demonstrate that most proteases will stimulate quiescent cells
to proceed through a sequence of events leading to a round of
cell division (46–56). However, to date, only chick embryonic
fibroblasts and some (46, 55), but possibly not all (53), mouse
3T3 cell lines have been found to respond to protease stimula-
tion. Other cell types are unresponsive (53, 55).

Invariably, studies involving the treatment of fibroblasts with

purified proteases have used cultures of low cell density ren-
dered quiescent by serum deprivation. The growth-stimulatory
potential of added proteases has thus been studied with respect
only to the ability to overcome a serum block, thereby initiating
a single round of cellular multiplication. The potential roles of
proteases have yet to be assessed in two of the most important
growth characteristics of the transformed phenotype: the ability
to overcome density-dependent growth regulation (high cell
density in excess serum), and the ability to grow in semisolid
medium. However, the ability of a cell to grow in semisolid
medium has been observed recently to correlate with the dis-
organization of cytoplasmic actin-containing bundles; exog-
enous proteases facilitate this in normal fibroblasts (57).

MATERIALS AND METHODS

Trypsin, 2× crystalline, was from Nutritional Biochemicals
Corp. and Pronase was B grade, 45,000 PUK/g, from Calbio-
chem. Highly purified bovine plasmin was a kind gift from K.
Fugikawa. Beef pancreatic trypsin inhibitor (Type I-P; 1 mg
inhibits 3.1 mg of trypsin) and soybean trypsin inhibitor (Type
I-S; 1 mg inhibits 1.5 mg of trypsin) were from Sigma Chemical
Co. Diisopropylfluorophosphate was also donated by K. Fugi-
kawa. To prepare the modified trypsin, it was added to a 5
mg/ml solution of trypsin to a final concentration of 5 mM in
0.05 M Tris at pH 8.0, with incubation for 2 hr at 25° and ex-
haustive dialysis against distilled water at 4°.

Baby hamster kidney cells (BHK) and polyoma virus-trans-
formed cells (PY BHK), clone WT-1, were generously provided
by W. Eckhart. Cells were maintained at subconfluent densities
at 37° in a 10% CO_2 atmosphere in Dulbecco's modified Eagle's
medium (DEM) containing 10% (vol/vol) fetal calf serum from
a single lot (North American Biologicals, Inc.). New cultures
were initiated from frozen stocks approximately every 2
months; changes in cellular properties were never observed
during this period in culture. Stock cultures were negative in
tests for mycoplasma contamination (kindly performed by G.
Kenny of the University of Washington).

RESULTS

The culture assays usually used to characterize cellular growth
properties require 1–3 weeks for completion. Because of the
instability of most enzymes, these lengthy periods have seriously
limited the feasibility of studying the effects of purified en-
zymes on the growth parameters defined in these classic assays.
In an attempt to overcome this limitation, we have approached
steady-state cultural conditions by a protocol of changing the
medium on a daily basis. This regimen has an additional ad-
vantage over infrequent feeding in that growth may be regu-
lated more uniformly by the availability of serum growth fac-
tors and by topological constraints (58, 59) rather than by nu-
trient exhaustion or by the accumulation of toxic products in
the medium (59–61). In each experiment, we tested the pro-

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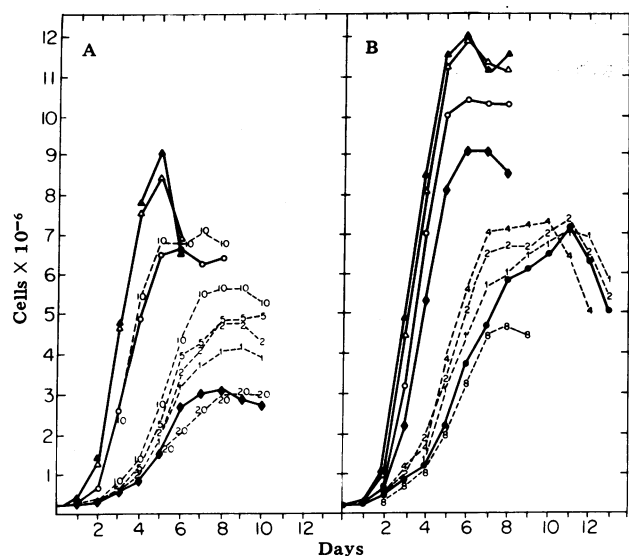
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FIG. 1. Growth of BHK (A) and PY BHK (B) as a function of serum and trypsin concentration. Cells were grown at 37° with daily medium changes of DEM having serum concentrations of 1% (●), 4% (♦), 10% (O), 30% (△), or 50% (▲). BHK in 4% and 10% serum and PY BHK in 1% serum were daily supplemented with fresh trypsin to the final concentrations (μg/ml) indicated by the small numbers used as experimental points (- - - -); some cultures were without trypsin (—). Cells were seeded at 2×10^5/3-cm plastic petri plates (Lux) in 2 ml of DEM containing the indicated amount of dialyzed fetal calf serum (1 volume dialyzed against 9 volumes of DEM for 24 hr at 4° and stored frozen at −20°). Cell number, given as the total per 3-cm petri plate (about 8.7 cm^2 growth area), was determined by a Coulter counter after dispersion of the cells in 0.25% (wt/vol) trypsin.
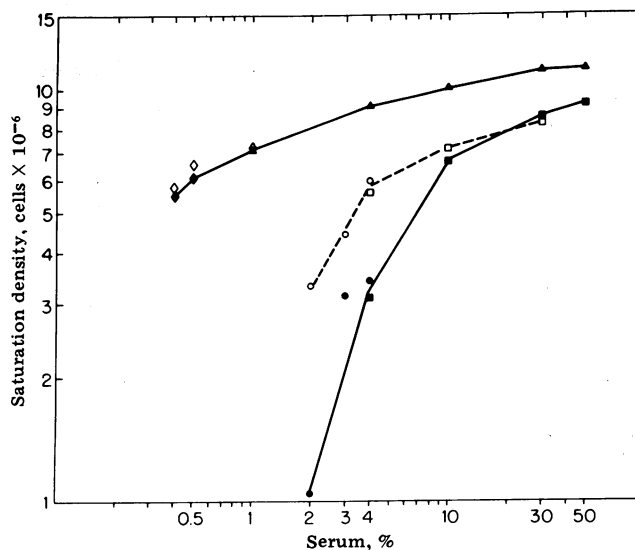

FIG. 2. Maximal effects of trypsin on the saturation densities of BHK and PY BHK as a function of serum concentration. Saturation densities (maximum cell number per 3-cm dish) as a function of serum concentration (—) are shown in two independent experiments for BHK (■, ●) and PY BHK (♦, ▲). Corresponding enhanced saturation densities at optimal trypsin concentrations (- - - -) are given for BHK (□, O) and PY BHK (◊, △). Saturation densities and optimal trypsin concentrations were determined as shown in Fig. 1.

tease(s) over a broad range of concentrations including those resulting in cell detachment or toxicity. In this fashion, an optimal protease concentration for producing the maximal effects has been determined for each experimental situation.

Saturation Density and Serum Requirement. Typical growth curves for normal and transformed BHK cells growing in liquid medium are shown in Fig. 1. At serum concentrations in excess of about 30%, both BHK and PY BHK reached maximal saturation densities under the defined growth conditions. In other experiments (not shown) we tested serum concentrations up to 100% without increasing the saturation density of either BHK or PY BHK above the levels shown here. Fig. 1 clearly shows that the maximal saturation density of PY BHK cells is distinctly higher than that of the untransformed parent BHK cells.

At serum concentrations below 30%, growth became increasingly serum-dependent. Fig. 1 shows that, as expected, BHK had a much higher serum requirement than PY BHK and also illustrates the effect of adding different amounts of trypsin to fixed serum concentrations. With low serum concentrations, the saturation density of BHK, but not PY BHK, was markedly elevated by the presence of trypsin in the culture medium. However, at high serum concentrations, the trypsin effect was not observed. These findings are extended in Fig. 2 which is a composite representation of several individual experiments.

In addition to lower saturation densities, BHK and PY BHK grown in limiting serum concentrations displayed lower growth rates and longer lag times preceding active multiplication in freshly seeded cultures. At low serum concentrations, trypsin generally enhanced the growth rates of both BHK and PY BHK but had no effect on the lag times.

Adhesion and Morphology. BHK cells growing in low serum concentrations with trypsin at concentrations optimal for growth stimulation were much more easily detached from the

plastic substrate than were cells from untreated cultures. With respect to adhesive properties, trypsin-stimulated BHK cells are thus more like transformed cells than are untreated BHK cells.

At trypsin concentrations optimal for growth stimulation, it was notable that reproducible morphologic changes were not observed. However, at about twice the optimal trypsin concentrations, BHK cultures displayed large numbers of rounded cells and overall had morphologic features similar to those of untreated cultures of PY BHK. At these trypsin concentrations, BHK cells continued to multiply but, if the trypsin concentration was further increased, there was a rapid shift toward a state in which the cells became completely detached from the substrate and multiplication ceased (Fig. 3).

Proteolytic Activity in the Culture Medium. A possible explanation for the failure of trypsin to enhance the maximal saturation density of BHK, or the saturation density of PY BHK in either low or high serum concentration, is that under these particular conditions (high serum or the presence of transformed cells) proteolytic activity could be uniquely inhibited or inactivated. Dose-response curves showing the effect of trypsin on the saturation density of normal and transformed BHK cells are shown in Fig. 3. There was no stimulatory effect on the saturation density of PY BHK in 1% serum or on BHK in 30% serum, but at sufficiently high concentrations, proteolytic activity was manifested in the detachment of the cell sheet. Thus, selective inhibition is not a likely explanation for the observed differential trypsin effects on growth.

We have also directly measured the proteolytic activity in trypsin-supplemented culture media by means of the casein assay (Table 1). At serum and trypsin concentrations optimal for the growth stimulation of BHK there remains ≤0.5% of the caseinolytic activity measured in the absence of serum. This residual activity declines about 50% over a 24-hr period at 37°. The level of activity and its rate of decline are independent of whether or not cells (either BHK or PY BHK) are present. To achieve a similar level of caseinolytic activity in higher concentrations of serum it is necessary to increase the trypsin concentration proportionally. Nevertheless, the rate of decline
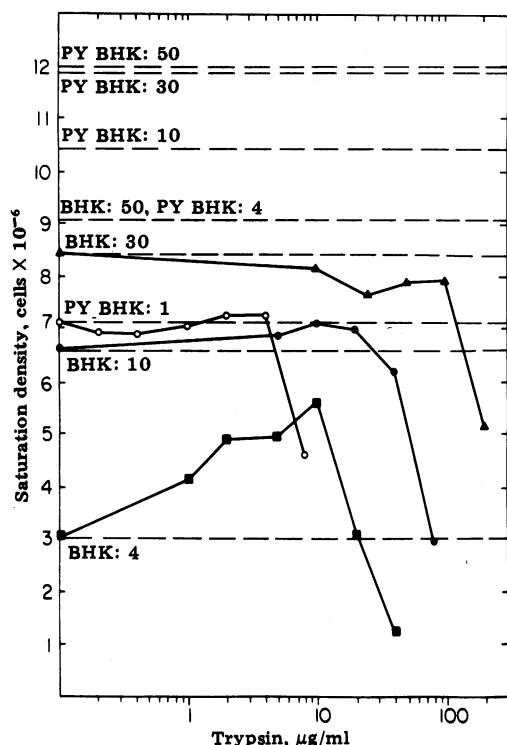
FIG. 3. Saturation densities of BHK and PY BHK as a function of serum and trypsin concentration. Saturation densities (maximum cell number per 3-cm dish) as a function of serum concentration are shown as dashed lines (— —) and are designated with the appropriate cell type and the serum concentration (%) used. The effect of trypsin on saturation density (—) is shown for BHK in 4% serum (■); BHK in 10% serum (●); BHK in 30% serum (▲); and PY BHK in 1% serum (○). Saturation densities were determined as shown in Fig. 1.

Table 1. Differential inhibition of proteolytic and growth-stimulating activities of trypsin

Experimental condition*	Relative proteolytic activity,† %	Relative growth stimulation,‡ %
Control	100	100
BPTI	<0.01	35 (105)§
STI	<0.01	97 (46)¶
DFP-treated	0.08	25

* Trypsin at final concentration of 10 μg/ml was assayed for growth stimulation in the presence or absence of bovine pancreatic trypsin inhibitor (BPTI) at 5 μg/ml; trypsin at 5 μg/ml was tested in the presence or absence of soybean trypsin inhibitor (STI) at 50 μg/ml. Trypsin treated with diisopropylfluorophosphate as described in *Materials and Methods* was tested for growth stimulation at 10 μg/ml. Dilutions of these preparations were used to determine the proteolytic activity of each.
† Proteolytic activity was determined in the absence of serum by comparing the rate of casein hydrolysis of the sample being tested relative to that with known amounts of trypsin. The samples to be tested and standard trypsin solutions were diluted serially into 1% crude casein (Sanalac nonfat powdered milk) in 0.1 M Tris, pH 8.0. After incubation at 37°, the optical density was read at 500 nm. Activity was determined from a standard curve obtained by plotting the change in optical density versus trypsin concentration.
‡ Growth stimulation was determined in the standard growth assay for BHK in 5% serum as described in Fig. 1. In the test of STI, treated and untreated cultures were grown in 4% serum. Stimulation is given in terms of the percentage of the increase in saturation density due to native trypsin alone.
§ Because BPTI by itself slightly lowers the saturation density, the number in parentheses represents the value of stimulation obtained if the saturation density of trypsin plus BPTI is compared to that of BPTI alone.
¶ Because STI by itself slightly elevates the saturation density, the number in parentheses represents the value of stimulation obtained if the saturation density of trypsin plus STI is compared to that of STI alone.

of caseinolytic activity in medium containing a high serum concentration is approximately the same as that found in medium containing a low serum concentration. These findings provide further evidence against the possibility of selective inhibition and also confirm observations on morphology and adhesion, such as described earlier, that suggest that considerable proteolytic activity exists over the full 24 hr between medium changes.

Growth in Agar. The original soft agar assay (14) was modified by adding an overlay consisting of an equal volume of liquid medium above the agar layer. The liquid medium was changed on a daily basis. The effect of trypsin on the growth of BHK and PY BHK is shown in Fig. 4. As the trypsin concentration was increased, there was an initial elevation of the growth rate of BHK, and then the effect became toxic. However, trypsin had no stimulatory effect on the final plating efficiency or on the average size (data not shown) of the colonies that appeared. In contrast to BHK, PY BHK grew efficiently in agar. Trypsin also had no effect on the plating efficiency or colony size of PY BHK in agar.

Protease Specificity and Function. At optimal levels, Pronase will stimulate the growth of BHK in low serum to the same extent as optimal trypsin levels. Indeed, all of the effects attributed to trypsin are also observed with Pronase. In the experiments described in this communication, we used a highly purified trypsin preparation that displays a single high molecular weight band upon electrophoresis in sodium dodecyl sulfate gels. The optimal stimulatory concentration of this preparation in 4% serum was 10 μg/ml while that of Pronase was only 2 μg/ml despite the fact that the Pronase preparation is crude and contains many protein species. The lower optimal concentration of Pronase may reflect that, unlike trypsin,

Pronase is not effectively complexed by serum protease inhibitors (48, 62).

Highly purified plasmin was only 29% as active as trypsin or Pronase in enhancing the saturation density of BHK in 4% serum. Plasmin also displayed optimal activity over a broader range of concentrations (0.2–1.0 μg/ml). These differences may in part be explained by the relative instability of plasmin (63) and by the fact that plasmin interacts differently with serum protease inhibitors than does trypsin (6, 64).

In the range of 2–4% serum, the maximal effects of trypsin, Pronase, and plasmin on growth were approximately equivalent to increases in serum protein representing 200, 1000, and 2500 times the mass of these three proteases, respectively, at their optimal concentrations (Fig. 2). These relative activities may be underestimated, especially in the case of trypsin, because serum protease inhibitors may to some extent inactivate growth-stimulatory activity. Nevertheless, these considerations suggest that proteases possess a high degree of stimulatory activity relative to that of bulk serum protein.

Further evidence indicative of specificity in protease-induced growth stimulation derives from analysis of several trypsin preparations ranging from very crude to highly purified. These different preparations vary by as much as 15-fold in specific proteolytic activity. We found that the optimal growth-stimulatory concentration of each of these preparations was inversely proportional to the specific proteolytic activity. This suggests that the mitogenic activity in these preparations "co-purifies" with trypsin and is probably associated with the
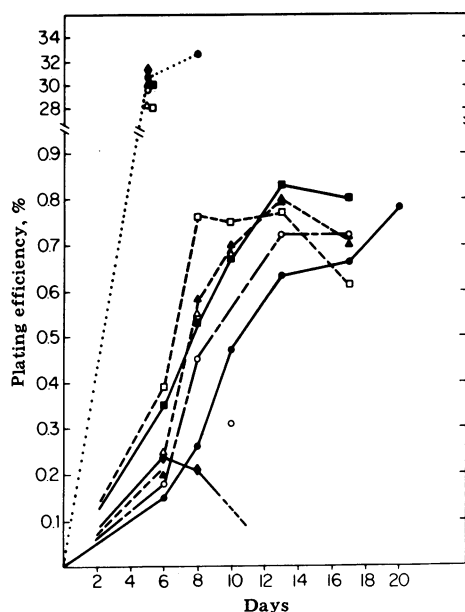
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FIG. 4. Effect of trypsin on the plating efficiency of BHK and PY BHK cells in agar. BHK (—, - - - -, — - —) and PY BHK (· · · ·) were suspended in agar under liquid medium at 37°; the liquid medium was replaced daily with 5 ml of liquid DEM (10% fetal calf serum) containing freshly added trypsin to final concentrations of 0 μg/ml (●), 12.5 μg/ml (O), 25 μg/ml (▲), 37.5 μg/ml (△), 50 μg/ml (■), 100 μg/ml (□), and 200 μg/ml (♦). Freshly prepared 5-cm base plates, each containing 4 ml of 0.55% agar with DEM and 10% fetal calf serum, with or without trypsin, were overlaid with 10^5 PY BHK or 10^6 BHK cells in 1.5 ml of 0.36% agar with DEM and 10% serum, with or without trypsin. Plating efficiency was expressed as the percentage of the plated cells giving rise to colonies larger than 103 μm in diameter as determined microscopically with a calibrated grid reticle.

trypsin molecule itself. As mentioned, the purest trypsin preparation showed a single band upon gel electrophoresis.

Do proteases mediate growth stimulation via direct proteolytic activity? We attempted to answer this question with trypsin because trypsin has been intensively studied with respect to the function and inactivation of the active site of the molecule. Table 1 documents our findings with three classic means of trypsin inactivation. In every case the caseinolytic activity of trypsin was essentially abolished while the growth-stimulatory activity was only moderately reduced.

DISCUSSION

We have found that the primary effect of trypsin, Pronase, and plasmin on the growth of normal cells is to lower the serum requirement. However, trypsin does not enhance growth in conditions of excess serum or when cells are suspended in soft agar. Thus, trypsin has no effect on two important growth characteristics used to differentiate normal and transformed cells *in vitro*.

In this investigation we concentrated much of our effort on the study of trypsin. Trypsin is easily obtained highly purified, it is relatively stable, and its properties have been extensively documented. Furthermore, in previous studies, trypsin has generally been at least as active as any other protease tested with respect to the specific growth-stimulatory activity of the enzyme and the maximal growth response attained (51, 53, 54, 56) and also in the dissolution of cytoplasmic actin-containing bundles (57). Notably, this generalization includes the two major potential serum proteases, plasmin (51, 54, 57) and thrombin (54, 56, 57). The fact that trypsin fails to increase the plating efficiency of BHK cells in agar is of special interest because growth in semisolid medium may be diagnostic of oncogenicity

(8, 9, 14–22) and because it is reputed to be a direct result of the exogenous protease, plasmin (5, 8, 12, 13). Because trypsin and plasmin are both serine proteases having similar specificities, our results suggest that plasmin might not be sufficient, or perhaps even necessary, for the growth of these cells in semisolid medium. However, it also is possible that plasmin has a unique physiological specificity that is lacking in trypsin.

The maximal effects of trypsin, Pronase, and plasmin in reducing the serum requirement are equivalent to a reduction of serum protein equal to 200, 1000, and 2500 times the mass of these three proteases, respectively, at their optimal concentrations. In published studies containing sufficient data to allow comparison of the mitogenic potential of unfractionated serum with that of proteases one can calculate values similar to ours for growth-stimulatory activity in terms of serum mass equivalents—i.e., 500 for thrombin (49), 800 for trypsin (65), 200–300 for trypsin (51), 250–350 for Pronase (55), and 150 for plasmin (51). However, from Fig. 2 it can be estimated that, under optimal conditions for protease stimulation, the effect of viral transformation on the serum requirement of BHK cells is about 10-fold greater than the maximal effect of protease.

The results of previous studies are in accord with our conclusion that proteases effect a reduction in the serum requirement of normal cells. Prior to this study, the protocol used in investigating the effects of purified proteases has always involved cultures of low cell density rendered quiescent by serum deprivation (46, 47). Moreover, data exist for both 3T3 cells (55) and chick embryo fibroblasts (51) that are consistent with the idea that protease treatment only partially substitutes for serum in growth processes and that the effect of viral transformation is probably much greater. The failure of exogenous proteases to account fully for the effects of viral transformation on the serum requirement may reflect the artificiality of the test systems or that factors other than proteases may be involved in this alteration.

There is substantial evidence that proteases mediate growth stimulation via direct proteolytic activity (46–49, 52, 53) and that the site of mitogenic activity is cellular and not a component of the medium (46–48, 54). It has been further suggested that the locus of proteolytic stimulation is on the cell surface rather than at an intracellular site (46, 48), but this has yet to be convincingly demonstrated. Among the small number of proteins that are cleaved by mitogenic concentrations of various proteases, no polypeptide has yet been detected which is necessary or even sufficient for growth stimulation (3, 50, 54, 56). Recent reports have shown that trypsin is actually taken up by cells and may exhibit intracellular activity (66, 67). Thus, the site of protease action in the process of growth stimulation has yet to be clarified.

In contrast to previous studies, we found that direct proteolytic activity does not seem to be primarily responsible for the mitogenic effects observed. With trypsin we used specific macromolecular inhibitors and diisopropylfluorophosphate under conditions almost identical to those reported by others (46–48, 52, 53), yet with contrasting results. In every case, we repeatedly found that, whereas the proteolytic activity of trypsin was abolished as expected, growth-stimulatory activity was only partially decreased, if at all.

An alternative to the possibility of a dissociation of growth-stimulatory activity from proteolytic activity on the trypsin molecule is that mitogenic activity might reside in tryptic fragments or peptides in the trypsin preparations. Trypsin fragments are known to occur in almost all highly purified commerical preparations and some of these have been found to retain esterolytic activity while displaying altered sensitivity to certain active-site inhibitors (68). On the other hand, there

are also many examples of peptides and polypeptides that are devoid of hydrolytic activity but exhibit potent growth factor activity (59). In fact, it has been recently suggested that all serum growth factors may be polypeptide hormones or hormone-like materials (59, 69).

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