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Transformation-enhancing activity of gelatin-binding fragments of fibronectin

(cathepsin G/malignant transformation/plasma cryoprecipitates/plasmin/Rous sarcoma virus)

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ABSTRACT **Studies have established that cryoprecipitates of the plasma of tumor patients contain a biological activity enhancing morphological cell transformation (transformation-enhancing factor; TEF) in cultures of chicken embryo fibroblasts infected with temperature-sensitive mutants of Rous sarcoma virus. We report here that similar TEF activity is effected by defined fragments of human plasma fibronectin obtained by limited digestion with major humoral or tissue proteinases. TEF activity was obtained from plasminolytic fragments of fibronectin and from cathepsin G-treated fibronectin. No activity was recorded from intact dimeric fibronectin or its reduced and alkylated subunits, from fibrinogen or its plasminolytic fragments, or from plasmin (EC 3.4.21.7) or cathepsin G (EC 3.4.21.20) treated or untreated with proteinase inhibitors. All of the TEF activity of the proteolytic fragments of fibronectin was located on the gelatin-binding peptides. The minimum effective doses in the TEF assay were 750 ng/ml of plasmin-treated fibronectin, 100 ng/ml of gelatin-binding plasminolytic fibronectin (enriched in M_r 180,000–190,000 polypeptides), and 100 ng/ml of gelatin-binding fragments of cathepsin G-treated fibronectin (enriched in a M_r 30,000 fragment). TEF activity of proteinase-treated fibronectin was inhibited by gelatin and by intact dimeric fibronectin. The potent TEF activity of proteolytic fragments of fibronectin raises the possibility that they may have a role in malignant transformation.**

Transformation-enhancing factors (TEF) have been shown to be present in the culture media of tumor virus-transformed cells and of spontaneously transformed established cell lines. TEF activity is detected as promotion of the morphological transformation of chicken embryo fibroblasts infected by Rous sarcoma virus or its temperature-sensitive mutants (1, 2). Moreover, TEF activity has been found *in vivo* in human plasma cryoprecipitates from patients affected with different neoplastic diseases (leukemia, lymphoma, and solid tumors) but not in healthy controls or in patients with nonneoplastic diseases (3). TEF activity in plasma cryoprecipitate appears to have potential diagnostic and prognostic value (4). In some tumor patients, TEF activity is detectable in whole plasma, but fractionation of plasma into the cryoprecipitate and its supernatant is an effective means to separate TEF activity (which is greatly enriched in the cryoprecipitate) from transformation-inhibiting factors (TIF) (which are mainly detected in the supernatant) (3).

Although the molecular identities of TEF and TIF activities have not been defined, it has been proposed that proteins involved in blood coagulation may posses TIF activity, whereas enzymes and breakdown products of fibrinolysis may posses TEF activity (3). That TIF activity may be assigned to some of the proteins involved in blood coagulation has been demonstrated (5). In this study, we tested components and products of fibrinolysis for TEF activity. Among the reasons that led us to look for TEF activity in fibronectin degradation products were the findings that plasma fibronectin is known to coprecipitate with fibrinogen in the cold (6, 7), that loss of pericellular matrix fibronectin is associated with malignant transformation of mesenchyme-derived cells (8), that secretion of plasminogen activator is associated with malignant transformation and invasive growth (9, 10), and that fibrin (or fibrinogen, or both) split products are found in circulation in malignancies (11, 12). The results of the present study show that potent TEF activity is associated with defined fragments of purified human plasma fibronectin digested with plasmin (EC 3.4.21.7) or with the major tissue proteinase cathespin G (EC 3.4.21.20). In contrast, fibrinogen or its degradation products had no TEF.

MATERIALS AND METHODS

Fibronectin and Its Proteolytic Fragments. Fibronectin was purified from human plasma by affinity chromatography under nondenaturing conditions as described (13). In $NaDodSO_4$/polyacrylamide gel electrophoresis, the purified chemically reduced protein showed, depending on the amount applied, either a doublet of polypeptide bands or a single band of M_r 220,000. In two-dimensional immunoelectrophoresis against polyvalent antibodies to human plasma proteins, the purified fibronectin gave only one immunoprecipitate arch, which was identical with that obtained with anti-fibronectin antibodies. The purified fibronectin did not show any contamination with fibrinogen or plasma transglutaminase as judged by immunoelectrophoresis against specific antisera. Reduced and alkylated fibronectin was prepared according to Konigsberg (14) by using 8 M urea/10 mM dithiothreitol/100 mM *N*-ethylmaleimide. Before analyses, the preparation was dialyzed against 1 mM phosphate buffer (pH 6.8).

The plasminolytic digestion of fibronectin was performed by mixing the purified protein with human plasmin (15 casein units/mg; Kabi, Stockholm, Sweden) or porcine plasmin (Sigma) in 0.1 M Tris·HCl (pH 8.0). Both preparations were used at an enzyme/substrate ratio of 1:20 (wt/wt). During incubation at 37°C, samples were taken at various intervals, and digestion was stopped by addition of diisopropyl fluorophosphate to a 10-fold molar excess over plasmin. The samples were dialyzed against 0.01 M sodium phosphate/0.14 M NaCl, pH 7.5, for 3 days at room temperature with six changes of buffer.

Fibronectin was also digested with cathepsin G (15). This enzyme was a gift from J. Saklatvala and P. Starkey (Strangeways Research Laboratory, Cambridge, England), obtained from a

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Abbreviations: FE, factor of enhancement; TEF, transformation-enhancing factors; TIF, transformation-inhibiting factors.

patient with chronic myeloid leukemia. It had been purified from neutrophil leukocytes by a modified procedure based on that of Baugh and Travis (16). The preparation of cathepsin G had an activity of 350 azocasein units/mg and gave under reducing conditions a M_r 27,500–30,000 band in $NaDodSO_4$/polyacrylamide gel electrophoresis. Digestion of fibronectin with cathepsin G was carried out as described (17) by using an enzyme/substrate ratio of 1:200 and incubation for 16 hr at 37°C. Phenylmethylsulfonyl fluoride (5 mM) was used to stop the reaction. Dialysis was against P_i/NaCl as before.

To separate gelatin-binding fragments from other peptides, the proteolytic digests were mixed with 1 vol of 50% (wt/vol) gelatin-Sepharose, prepared as described (13), in 50 mM Tris·HCl, pH 7.5/5 mM benzamidine/0.02% sodium azide. The mixture was rotated in an end-over mixer for 6 hr at room temperature and then applied to a column (10 × 0.8 cm); after stabilization of the column bed, the flow-through was collected (gelatin-nonbinding fraction). The elution of the gelatin-binding fraction was carried out with 1 M arginine (13) in the Tris/benzamidine/azide buffer. Both fractions were dialyzed at room temperature against P_i/NaCl before analysis.

Fibrinogen and Its Plasminolytic Fraction. Commercially available human fibrinogen (Kabi) was purified by passing it through gelatin-Sepharose as above, but this time to remove the contaminating fibronectin. The preparation gave, when chemically reduced, a triplet of bands corresponding to the α, β, and γ chains of fibrinogen in $NaDodSO_4$/polyacrylamide gel electrophoresis and reacted in double-diffusion precipitation tests with anti-fibrinogen but not with anti-fibronectin. The plasminolytic digestion of purified fibrinogen was performed in the same experimental conditions as described for fibronectin. The digest, according to analysis by $NaDodSO_4$/polyacrylamide gel electrophoresis in reducing conditions, was enriched in the fibrinogen fragments D and E (18).

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was by the method of Laemmli (19) with vertical slab gels. The acrylamide concentration was 3.3% (wt/vol) in the spacer gel and 10% in the separating gel as indicated. Unless otherwise specified, the samples were reduced with 10% (wt/vol) 2-mercaptoethanol in Laemmli's sample buffer. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 (20). Purified human plasma fibronectin and commercially obtained high and low M_r markers (Pharmacia, Uppsala, Sweden) were used as standards.

Assay for TEF Activity. TEF assay was as described (3). Secondary cultures of chicken embryo fibroblasts were infected in suspension with the temperature-sensitive mutant PA 1 (21) of the Schmidt–Ruppin strain of Rous sarcoma virus, at a low multiplicity of infection to give about 100 focus-forming units per 3×10^5 cells seeded per 8-cm^2 plate in medium 199 supplemented with 5% (vol/vol) newborn calf serum, 10% (vol/vol) tryptose phosphate broth, and antibiotics. The liquid medium was replaced 24 hr after seeding by semisolid medium containing 0.6% agar. After 5 days of incubation at 37°C, when foci of morphologically transformed cells became detectable, the cultures were shifted to 41°C (nonpermissive temperature for transformation) for 48 hr. At this time, when no foci were detectable, the gel medium was removed and replaced with liquid medium 199 containing 5% decomplemented (incubated for 30 min at 56°C) newborn calf serum, 10 μg of cycloheximide per ml, and the appropriate concentration of the sample to be tested or corresponding control preparation. (Use of the inhibitor of protein synthesis, cycloheximide, is not mandatory but gives more reproducible results in the TEF assay.) Immediately afterwards, the cultures were shifted to 37°C for 4 hr, observed microscopically, and stained supravitally with methylene blue, which selectively stains foci of virus-transformed cells (22). The number of foci were calculated in each plate, and TEF activity was recorded as the factor of enhancement (FE)—the ratio of the number of foci scored in triplicate test cultures to that in triplicate control cultures. Evaluation of the standard error and the statistical significance of the FE were calculated as described (23).

Other Methods and Reagents. Double-diffusion precipitation tests were made on 1% agarose plates by the method of Ouchterlony (24). The protein concentrations were assayed as described by Lowry *et al.* (25) with bovine serum albumin as standard. Gelatin (type I, porcine) and cycloheximide were from Sigma. Activated transglutaminase from human placenta was from Behringwerke (Marburg, Federal Republic of Germany). In analysis by $NaDodSO_4$/polyacrylamide gel electrophoresis, the enzyme was >95% homogenous. One unit of transglutaminase corresponds to the enzyme activity present in 1 ml of freshly pooled whole human plasma by the enzyme assay of Folk and Cole (26).

RESULTS

TEF Activity of Plasminolytic Fragments of Fibronectin. Fibronectin was digested with human plasmin at 37°C with an enzyme/substrate ratio of 1:20. The cleavage was monitored by $NaDodSO_4$/polyacrylamide gel electrophoresis. The polypeptide pattern obtained after different times of incubation shows (Fig. 1) the rapid digestion of fibronectin into M_r 31,000 and M_r 180,000–190,000 peptide chains and their further fragmentation, in agreement with a previous report (27). Essentially similar results were obtained when porcine plasmin was used to digest fibronectin. Aliquots of the same digestion were also tested in TEF assay. Significant activity was found to be associated with the fragment mixtures obtained after 10 min, 30 min, 3 hr, 6 hr, and 24 hr of incubation time (Table 1). Control

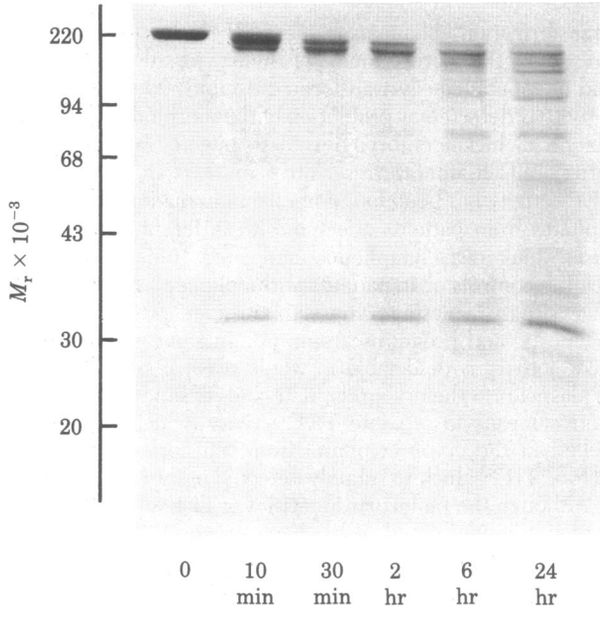

FIG. 1. Kinetic analysis of digestion of human plasma fibronectin by plasmin, with $NaDodSO_4$/10% polyacrylamide slab gel electrophoresis. In the digestion the enzyme/substrate ratio was 1:20 (wt/wt) with conditions as described. The samples analyzed correspond to 30 μg of fibronectin.

Table 1. TEF of proteolytic digests of fibronectin and of controls

Digests*			Protein concentration,	TEF assay†	
Protein	Enzyme	Digestion time	μg/ml	FE ± SEM	*P*
Fibronectin	Plasmin	10 min	15	1.9 ± 0.2	<0.001
		30 min	15	2.6 ± 0.3	<0.001
		2 hr	15	2.4 ± 0.3	<0.001
		6 hr	15	1.7 ± 0.1	<0.001
		24 hr	15	2.5 ± 0.2	<0.001
Fibronectin	Cathepsin G	24 hr	15	2.2 ± 0.2	<0.001
Fibrinogen	Plasmin	5 min	15	1.2 ± 0.1	NS
		30 min	15	1.1 ± 0.1	NS
		3 hr	15	1.0 ± 0.2	NS
		24 hr	15	1.0 ± 0.1	NS
		Controls			
Fibronectin	—	—	5	0.8 ± 0.2	NS
			15	0.8 ± 0.2	NS
			30	0.7 ± 0.1	NS
			100	0.7 ± 0.1	NS
Reduced and alkylated fibronectin	—	—	5	1.0 ± 0.2	NS
			15	1.2 ± 0.2	NS
			30	1.0 ± 0.2	NS
Fibrinogen	—	—	15	0.9 ± 0.1	NS
Plasmin	—	—	1	1.0 ± 0.1	NS
			5	0.9 ± 0.1	NS
iPr_2PF-treated plasmin	—	—	1	1.3 ± 0.1	NS
Cathepsin G	—	—	0.1	0.9 ± 0.1	NS
			0.5	0.9 ± 0.1	NS
$PhMeSO_2F$-treated cathepsin G	—	—	0.1 units/ml	1.0 ± 0.1	NS
Plasma transglutaminase	—	—	0.01	1.1 ± 0.3	NS
			0.1	1.0 ± 0.2	NS
			1	0.5 ± 0.1‡	NS

* The digestions were carried out and terminated as described; diisopropyl fluorophosphate (iPr_2PF) was used for plasmin and phenylmethylsulfonyl fluoride ($PhMeSO_2F$) for cathepsin G.
† TEF activity is expressed as factor of enhancement (FE) ± SEM; *P*, probability; NS, not significant.
‡ The TIF activity, also seen here for plasma transglutaminase (FE < 1) has been reported (5).

samples, including intact fibronectin, diisopropyl fluorophosphate-treated plasmin, and buffer control, were all negative. Furthermore, the active digests had no apparent effect when added to uninfected control cultures. In order to avoid the interference with intact fibronectin, still present after short digestion, the fragment mixture obtained after 24 hr of incubation was chosen to study the dose dependence in TEF assay. Plasminolytic fragments of fibronectin were able to promote cell transformation at the lowest concentration of 750 ng/ml (Fig. 2*A*). On the other hand, intact fibronectin had no TEF activity in concentrations up to 100 μg/ml (see Fig. 4*B*). Furthermore, reduced and alkylated fibronectin was also devoid of TEF activity (Table 1).

To exclude the possibility that our fibronectin preparations were contaminated by minute amounts of biologically active fibrinogen or its degradation products, we tested preparations of purified fibronectin-free fibrinogen and its plasminolytic fragments in TEF assay. Neither fibrinogen nor its fragments, which included different kinds of fragments D and E, had any TEF activity (Table 1). Plasma transglutaminase, which under certain conditions is found in partially purified preparations of fibronectin (28), also gave negative results. In fact, in agreement with earlier findings (5), this enzyme inhibited virus-induced cell transformation (Table 1).

Location of TEF Activity to the Gelatin-Binding Fragments of Fibronectin. In an attempt to identify the active fragments, the plasminolytic digests were fractionated by using gelatin-Sepharose affinity chromatography. The gelatin-binding fraction was enriched in peptides of M_r 180,000–190,000, and the gelatin-nonbinding fraction was enriched in peptides of M_r 31,000 (Fig. 3). TEF activity was only present in the gelatin-binding fraction, which was still active at 100 ng/ml (Fig. 2*B*).

In another approach, the plasminolytic digest of fibronectin was mixed with gelatin and then tested in the TEF assay. Gelatin (1 μg) was able to block the effect of 15 μg of the digest (Fig. 4*A*). Gelatin in itself had no activity. Intact fibronectin, when tested in similar competition experiments, was also active but much less so (Fig. 4*B*).

Gelatin-binding fragments also were isolated from fibronectin digested with cathepsin G. This enzyme, purified from human leukocytes, was chosen because, unlike plasmin, it is known to produce well-defined, small-sized, gelatin-binding fragments (17). During digestion, gelatin-binding fragments of M_r 200,000, 64,000, and 40,000 appear sequentially (17), and after prolonged digestion the M_r 40,000 fragment is converted into a M_r 30,000 gelatin-binding fragment, as will be described in detail elsewhere. Fig. 3 gives the polypeptide pattern of gelatin-binding and gelatin-nonbinding fractions of such late digests. As shown in Fig. 2*C* the gelatin-binding fraction, mainly composed of the M_r 30,000 fragments (Fig. 3), was active at doses as low as 100 ng/ml. The gelatin-nonbinding fraction was negative in all doses tested (up to 30 μg/ml).

DISCUSSION

The present results show that proteolytic fragments of human plasma fibronectin are highly active in promoting virus-induced

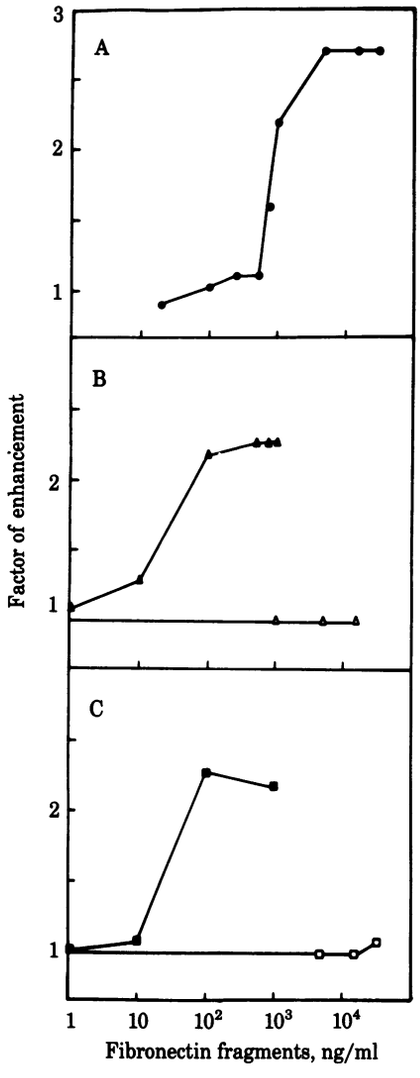

FIG. 2. TEF activity of proteolytic fragments of human plasma fibronectin. (*A*) Plasminolytic fragments (●). (*B*) Plasminolytic fragments separated into gelatin-binding (▲) and gelatin-nonbinding fractions (△). (*C*) Cathepsin G digests of fibronectin separated into gelatin-binding (■) and gelatin-nonbinding fractions (□). The activity is expressed as factor of enhancement in ratio to corresponding controls.

morphological cell transformation. All activity appeared to reside in the fragments binding to denatured collagen (gelatin). This conclusion is based on use of highly purified fibronectin and on lack of detectable activity in fibrinogen, its degradation products, or in plasma transglutaminase, which are potential contaminants of fibronectin preparations. Moreover, intact fibronectin, reduced and alkylated fibronectin, and the other control reagents, specifically plasmin, cathepsin G, and gelatin, had no activity. Our conclusion gets further support from the observation that gelatin in relatively small doses was able to block the TEF activity of the proteolytic fragments. It should be noted, however, that the TEF activity of the fragments is not necessarily due to the collagen-binding property of fibronectin, because this region of the molecule may contain other activities that have not been characterized as yet.

In addition to gelatin, we found that large doses of intact fibronectin and high doses of plasma transglutaminase were able to block the enhancement by the proteolytic fragments. Previously, by using another experimental system designed to test specifically for TIF activity, it was found that plasma transglutaminase and fibrinogen plus thrombin, but neither alone, were

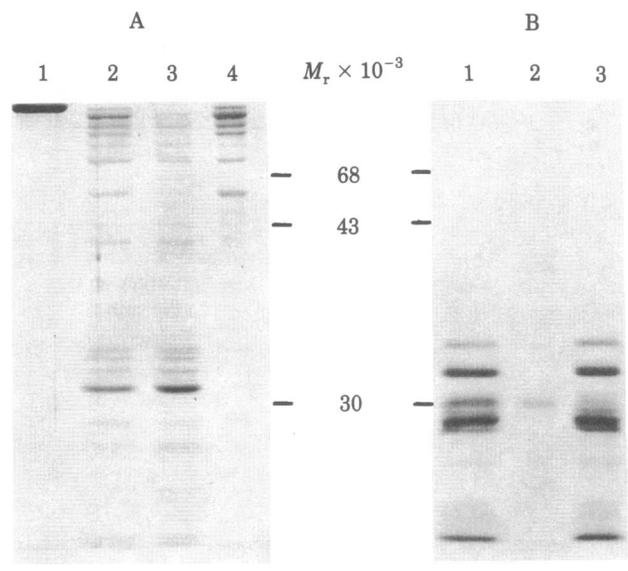

FIG. 3. Fractionation of proteolytic digests of human plasma fibronectin into gelatin-binding and gelatin-nonbinding fractions analyzed with $NaDodSO_4$/10% polyacrylamide slab gel electrophoresis. (*A*) Fifteen micrograms of intact fibronectin (lane 1), 24-hr plasminolytic digest of fibronectin (lane 2), its gelatin-nonbinding fraction (lane 3), and its gelatin-binding fraction (lane 4). (*B*) Cathepsin G digest (16 hr) of fibronectin (lane 1), its gelatin-binding fraction (lane 2), and its gelatin-nonbinding fractions (lane 3).

inhibitory (5). Both soluble and pericellular fibronectin are known to be substrates of plasma transglutaminase (28, 29), but whether fibronectin can augment the TIF activity of plasma transglutaminase is not known at present. However, the gelatin-nonbinding fraction, which presumably contains the transglutaminase site (27), had no TIF activity in the present assay.

We can only speculate about the mechanism of TEF activity of the gelatin-binding fragments of fibronectin. A plausible explanation would be that the dissociation of the different domains of intact fibronectin may give rise to fragments that have maintained an equal or increased affinity with one of the fibronectin interaction sites; the association of the fragment to one of the sites would prevent the binding of the intact fibronectin molecule, and the lack of the other domains in the fragment would prevent the assembly of a normal pericellular matrix.

The low doses of fibronectin degradation products (100 ng/ml or 3 nM) needed for TEF activity might suggest involve-

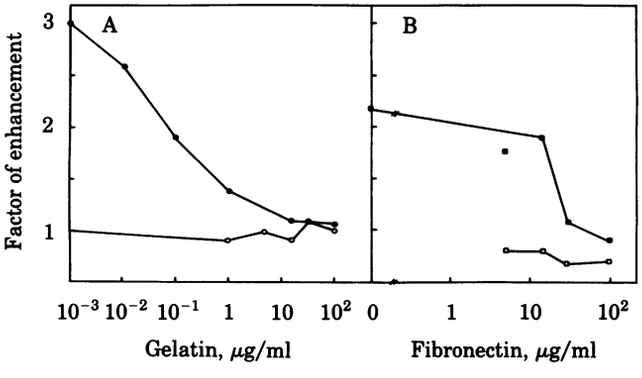

FIG. 4. Inhibitory effect of different concentrations of gelatin (●) (*A*) and of intact dimeric fibronectin (■) (*B*) on the TEF activity of plasminolytic fibronectin fragments used at a constant concentration of 15 μg /ml. The open symbols show the effects of gelatin (○) and intact fibronectin (□) alone.

ment of hormone-type or enzymatic mechanism of action. Because the digests were treated with diisopropyl fluorophosphate or phenylmethylsulfonyl fluoride and the effect was obtained in the presence of calf serum, it seems unlikely that the active fragments had serine-protease activity themselves. Moreover, no plasminogen-dependent caseinolytic activity has been found in the active fractions (unpublished observations). However, there is evidence, albeit indirect that plasminogen activator may act on substrates other than plasminogen and that these effects may be involved in virus-induced cell transformation (30). Furthermore, some of the fractions obtained from the plasma cryoprecipitate of cancer patients have both plasminogen activator and TEF activity (31). Thus, the possible relationship to plasminogen activators of (*i*) gelatin-binding fibronectin fragments and (*ii*) TEF activity in the culture medium of transformed cells (1) and in plasma cryoprecipitates of cancer patients (3) needs further study.

Since transformation-enhancing factors detectable by the present TEF assay are produced by virus-transformed cells in culture, the possible active role of fibronectin degradation products in the transformation process might be considered.

Elevated levels of fibrinopeptides (11) and of M_r 190,000 DNA-binding proteins recognized by fibronectin antibodies (32) have been detected in tumor patient plasma and serum, respectively. These findings have been thought to be due to the increase in coagulation disorders and in proteolytic events associated with malignancy. By considering that proteolytic fragments of fibronectin but not of fibrinogen show TEF activity *in vitro*, it seems possible that such fragments might be responsible, at least partially, for the TEF activity detected in plasma cryoprecipitate of tumor patients. That this indeed may be the case is suggested by our observation (unpublished data) that the TEF activity of the cryoprecipitates is selectively bound to gelatin-Sepharose.

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