Biochem. J. (1983) **210**, 137–144
Printed in Great Britain

Differences in polyamine availability and insertion into fibronectins released from normal and transformed cells

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(*Received 26 July 1982/Accepted 28 September 1982*)

1. Fibronectin released from transformed rat kidney cells compared with that released from normal rat kidney cells shows a 50% increase in amino group availability. 2. No such changes were observed in thiol and carboxy group availability or in sialic acid content. 3. The increased amino group availability is not due to a greater polyamine content, which was about 0.04 pmol/mg of protein. 4. Transglutaminase mediated the insertion of spermidine into normal cell fibronectin with linear kinetics. With fibronectin from transformed cells (temperature-sensitive mutant or wild-type), kinetics typical of substrate inhibition were observed. 5. Immunochemical analysis with an anti-polyamine antiserum and an anti-(human fibronectin) antiserum showed that fibronectins from normal and transformed cells react differently. The significance of these results is discussed in the light of changes in the secondary structure between the two fibronectins.

It is well documented that increases in polyamine levels accompany the growth of eukaryotic cells both *in vitro* and *in vivo* regardless of whether growth is normal or malignant (for recent reviews, see Jänne *et al.*, 1978; Quash & Roch, 1979; Campbell *et al.*, 1981; Heby, 1981). Their effect on growth may be related to: (1) the modulation of intracellular mechanisms such as the biosynthesis of DNA, RNA, proteins and lipids; (2) their interaction with membrane constituents.

This latter role was investigated by Quash *et al.* (1971), who provided evidence that anti-polyamine antibodies in the presence of complement were cytolytic for mammalian cells in tissue culture.

In the absence of complement, no cytolysis occurred, but transformed cells showed morphological modifications, whereas normal cells did not.

With the use of immunolatex spheres and ^{3}H-labelled antibodies, additional evidence was obtained for the existence of membrane putrescine sites (Quash *et al.*, 1978). This experimental approach suggested that the number or the accessibility of putrescine sites is modified with cell transformation and with the phase of the cell cycle.

Among the membrane proteins to which polyamines can be linked are the receptor sites for polypeptide hormones (Levitzki *et al.*, 1980; Yarden *et al.*, 1981), the histocompatibility antigens (Pober & Strominger, 1981) and fibronectin (Mosher, 1976). Several authors using fluorescent (dansyl) and radiolabelled polyamines have shown that the insertion of polyamines into proteins can be accomplished covalently into amide groups of protein-bound glutamine residues with the help of exogenous transglutaminase (Schrode & Folk, 1978; Lorand *et al.*, 1979).

Fibronectin is one of the cell-surface proteins that is released from the surface of transformed cells (Hynes, 1976; Olden & Yamada, 1977; Vaheri & Mosher, 1978; Ruoslahti *et al.*, 1981*a*). When present in the free state in plasma, it has been shown to be an effective acceptor of polyamines (Roch *et al.*, 1980).

It seemed reasonable to ask whether the fibronectin released from transformed cells, which has been shown to have a different sugar composition to that of normal cells (Wagner *et al.*, 1981), also had an altered polyamine content compared with that of normal cells.

This question was all the more pertinent since: (*a*) tumour cell fibronectin has been shown to have a different isoelectric point to that of normal cell fibronectin (Ruoslahti *et al.*, 1981*b*); (*b*) transglutaminase is preferentially localized in the membrane fraction of hepatoma cells compared with that of regenerating hepatocytes (Birckbichler *et al.*, 1976).

We therefore undertook a comparative study of fibronectins released into the culture medium from normal and transformed cells for their polyamine

0306-3283/83/010137-08$2.00

content, polyamine binding capacity and reaction with anti-polyamine antibodies.

Experimental

Materials

[^{14}C]Spermidine trihydrochloride {*N*-(3-aminopropyl)[1,4-^{14}C]butane-1,4-diamine trihydrochloride; sp. radioactivity 120 Ci/mol} and [^{14}C]-spermine tetrahydrochloride {*NN'*-bis-(3-aminopropyl)[1,4-^{14}C]butane-1,4-diamine tetrahydrochloride; sp. radioactivity 122 Ci/mol} were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. [^{14}C]Ethanolamine (sp. radioactivity 4.3 Ci/mol) was from New England Nuclear, Boston, MA, U.S.A. Exogenous transglutaminase (fibrogammine) from human placenta was a gift from Dr. P. Lafay (Lab. Hoechst-Behring, Paris, France). The enzymic activity of each batch was verified and 1 unit corresponds to the amount of enzyme capable of inserting 100 pmol of putrescine into casein for 2 h at 37°C.

The following materials were obtained commercially: (1-ethyl-3,3-dimethylaminopropyl)carbodiimide hydrochloride from Merck, Darmstadt, Germany; 5,5'-dithiobis-(2-nitrobenzoic acid) from Serva, Heidelberg, Germany; thrombin (30 units/ml) from Behringwerke A.G., Marburg, Germany; spermidine trihydrochloride, spermine tetrahydrochloride, phenylmethanesulphonyl fluoride (a proteinase inhibitor), cyclohexylaminopropanesulphonic acid, picrylsulphonic acid and *N*-acetylneuraminic acid were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium dodecyl sulphate was purchased from BDH, Poole, Dorset, U.K., thiobarbituric acid from Eastman Kodak, Rochester, NY, U.S.A., and Pico-Fluor 30 from Packard Intrument Company Inc., Downers Grove, IL, U.S.A.

Methods

Cells. The cells used were normal rat kidney cells LA31 NRK transformed by a temperature-sensitive mutant of the Prague strain of Rous Sarcoma Virus (LA31) (Chen *et al.*, 1977). Both cell lines were kindly supplied by Dr. P. K. Vogt. Eagle's minimum essential medium, containing glutamine as supplied by Grand Island Biological Co., Grand Island, NY, U.S.A. supplemented with 3% $NaHCO_3$, 10% tryptose phosphate broth from Difco Laboratories, Detroit, MI, U.S.A., and 10% foetal-calf serum, was used for the growth of all cells. Cells were incubated at the appropriate temperature in a humid atmosphere of air/CO_2 (19:1, v/v). LA31 NRK cells were seeded at a density of 1.5×10^6 cells/plastic flask (75 cm^2) and reached confluence after 4 days at 33°C. At this time, the medium was changed and half the cells were kept at 33°C (the permissive temperature), whereas the other half was transferred to 39°C (the non-permissive temperature). Normal rat kidney cells transformed by the wild-type strain of Rous Sarcoma Virus B77 were seeded at 1×10^6 cells/plastic dish (diameter 10 cm) and grown under identical conditions.

Purification of fibronectin. Fibronectin was purified from the culture medium by precipitation with spermine by the method of Vuento *et al.* (1980) with the following modifications. To 100 ml of cell culture medium was added phenylmethanesulphonyl fluoride to a final concentration of 0.4 mM in solution in acetone at 40 mM and the mixture was left for 30 min at room temperature. It was then extensively dialysed against 5 mM-Tris/HCl, pH 7.5, at 4°C (four changes of 50 vol.) and then incubated at 4°C with spermine (5 mM final concentration in 5 mM-Tris/HCl, pH 7.5) for 2 days. After centrifugation at 11400 *g* for 20 min and two washings with 5 mM-Tris/HCl, pH 7.5, containing 5 mM-spermine, the pellet was dissolved in 0.01 M-cyclohexylaminopropanesulphonic acid at pH 11 and extensively dialysed. The addition of [^{14}C]spermine to the incubation medium before dialysis and determination of radioactivity of the diffusate and the dialysis residue ensured that there was no residual spermine bound to fibronectin after prolonged dialysis. This approach yielded 40–80 μg of fibronectin/ml of culture medium.

When culture medium alone (without cells) was subjected to the same incubation temperatures and the same preparative procedure a maximum of 8 μg of fibronectin/ml was obtained.

Polyacrylamide-gel electrophoresis. Samples were treated with denaturing buffer (1% sodium dodecyl sulphate, 1% β-mercaptoethanol final concentrations) for 3 min at 90°C. Electrophoresis was carried out on a 7.5% polyacrylamide slab gel containing sodium dodecyl sulphate as described by Laemmli (1970). Gels were stained with Coomassie Blue. Proteins used for molecular-weight determination were RNA polymerase, bovine serum albumin and ovalbumin.

Chemical determination of the number of primary amine thiol and carboxy groups. Free amino groups were determined by their reaction with 0.1% picrylsulphonic acid in 4% $NaHCO_3$ buffer, pH 8.5, by the procedure of Habeeb (1966).

Available thiol groups of cysteine were determined by the method of Ellman (1959) with 5,5'-dithiobis-(2-nitrobenzoic acid) in 100 mM-phosphate buffer, pH 7.

Available carboxy groups were titrated by measurement of labelled amides formed with [^{14}C]-ethanolamine in the presence of (1-ethyl-3,3-dimethylaminopropyl)carbodi-imide hydrochloride by the method of Goodfriend *et al.* (1964).

Sialic acid determination. Sialic acid content was

determined by the method of Warren (1959) with thiobarbituric acid after hydrolysis of the sugar residues of fibronectin with 0.05 M-H_2SO_4 at 80°C for 1 h. *N*-Acetylneuraminic acid was used as a standard.

Assay of polyamine binding to fibronectin. To experimental tubes containing 50 μg of fibronectin were added [^{14}C]spermidine (sp. radioactivity 1 Ci/0.4 mol) in amounts ranging from 40 to 350 nmol. To the mixture was then added 60 μl of transglutaminase (10 units) and 60 μl of thrombin (0.48 unit) to ensure that all the transglutaminase added was in the active form. The reaction was initiated by the addition of calcium to a final concentration of 6 mM and completed to a final volume of 450 μl with 50 mM-NaCl/0.01 M-Tris/HCl, pH 7.5. One series of control tubes contained all the reagents as above except fibronectin, which was replaced by buffer. Another series contained all the reagents except calcium. After incubation for 4 h at 37°C with gentle shaking, to 300 μl of incubation medium was added 300 μl of ice-cold 20% trichloroacetic acid. After 16 h at 4°C, the tubes were centrifuged at 2400 *g* for 15 min. The precipitate was washed three times with 4 ml of 10% trichloroacetic acid containing 10 mM-spermidine until the supernatant was free of radioactivity. The last pellet was dissolved in 300 μl of 1 M-NaOH. The radioactive content of 100 μl was counted with 6 ml of Pico-Fluor 30 in a Packard liquid-scintillation spectrometer BPLD with an efficiency for ^{14}C of 85%. The number of nmol of spermidine bound per 50 μg of fibronectin was determined from the specific radioactivity of the polyamine as follows:

$$[(\text{Enzyme} + [^{14}\text{C}]\text{polyamine} + \text{fibronectin} + Ca^{2+}) - (\text{enzyme} + [^{14}\text{C}]\text{polyamine} + \text{fibronectin} - Ca^{2+})]$$
$$-[(\text{enzyme} + [^{14}\text{C}]\text{polyamine} + \text{buffer} + Ca^{2+}) - (\text{enzyme} + [^{14}\text{C}]\text{polyamine} + \text{buffer} - Ca^{2+})]$$

Each determination was carried out in duplicate and duplicates agreed within 3%. Non-specific binding in the absence of Ca^{2+}, whether for enzyme alone or enzyme + fibronectin, ranged from 0.12 nmol to 0.19 nmol over a spermidine concentration range of 90 μM to 780 μM. Specific binding to fibronectin over this same substrate concentration range was from 0.57 nmol to 0.84 nmol.

Polyamine content of acid-hydrolysed fibronectin. To determine the polyamine content of fibronectin, [^{14}C]spermine (sp. radioactivity 122 Ci/mol) was added to LA31 NRK cells at confluence in culture medium containing horse serum instead of calf serum. [This precluded any oxidation of the added spermine by spermine oxidase present in calf serum (Bachrach *et al.*, 1967)]. After an additional 5 h incubation, fibronectin in the culture medium was purified as described previously.

Of these fibronectins 250 μg was hydrolysed with 6 M-HCl for 16 h at 110°C in a sealed tube. After drying, the content of the ampoule was dissolved in 200 μl of 0.1 M-HCl and polyamines were analysed on an automatic amino acid analyser (Villanueva, 1982). A fraction collector was coupled to the exit of the fluorimeter and 400 μl fractions were collected for radioactivity determinations.

Immunological study of fibronectins. (*a*) Preparation of antisera. An antiserum antipolyamine was prepared in a goat by intravenously injecting the animal three times per week with acetylated lysozyme/spermine suspended in 0.14 M-NaCl buffered to pH 7.4 with 0.004 M-Tris/HCl (NaCl/Tris/HCl buffer). The initial dose of 140 μg was gradually increased to 10 mg over a period of 6 weeks and then kept at this level in subsequent inoculations (Quash & Jonard, 1967).

An anti-fibronectin antiserum was prepared in rabbits by injecting human plasma fibronectin purified as described by Vuento & Vaheri (1978). The homogeneity of the injected fibronectin was controlled by polyacrylamide-gel electrophoresis. Only one major band with a molecular weight around 210000 was found. Fibronectin (200 μg) was mixed with Freund's complete adjuvant and injected into the hind-foot pads of a rabbit. After 3 weeks, the animal received intravenous injections three times a week for 2 weeks of the antigen in doses increasing from 25 to 200 μg. At 1 week after the last injection, blood was taken by cardiac puncture.

All sera were clarified by centrifugation at 2400 *g* for 30 min just before use.

(*b*) Precipitin reactions between fibronectin extracted from culture medium of LA31 NRK at the permissive or non-permissive temperature and the appropriate antiserum. These were carried out by adding increasing quantities of fibronectin to constant amounts of antiserum (either 500 μl of anti-polyamine antiserum or 200 μl of anti-fibronectin antiserum). After 1 h at 37°C and 18 h at 4°C, the tubes were centrifuged (2500 *g* for 20 min) and washed twice with the NaCl/Tris/HCl buffer, pH 7.4. Each precipitate was dissolved in 350 μl of 0.1 M-NaOH and protein content was determined by the method of Lowry *et al.* (1951).

Results

To be able to compare the fibronectins released from normal and transformed cells without the attendant problems of differences due to the cell cycle, fibronectin was extracted from the culture

medium of LA31 NRK cells that had been grown to confluence at 33°C (permissive temperature) and then either maintained at this temperature or transferred to 39°C (non-permissive temperature). It was purified as described in the Experimental section and subjected to physical, chemical and immunochemical analyses.

Electrophoretic mobility on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate

It was found (Fig. 1) that there was no significant difference in the electrophoretic mobility of the fibronectin purified from the culture medium of either normal cells (LA31 NRK at 39°C) or transformed cells (LA31 NRK at 33°C). This provides evidence that the fibronectin from both cells has not undergone changes in molecular weight that can be detected by polyacrylamide-gel electrophoresis (around 200000). As minor differences in sugar content may not have been detected by polyacrylamide-gel electrophoresis, we next examined the content of one sugar residue, sialic acid, which has been shown by Yamada *et al.* (1977) to be the terminal sugar on fibronectin.

Sialic acid content

When this was examined, it was found that the fibronectin extracted from the culture medium of normal or transformed cells contained similar quantities of sialic acid (71–73 nmol/mg of protein). This suggests that the insertion of terminal sialic acid takes place normally on the fibronectin molecule from both cell types. This would not have been the case if the sugar residues of the glycosidic chains with terminal sialic acid had been prematurely terminated.

In view of the similarity in molecular weight and sialic acid content, we tried to determine whether there were any differences in structure.

End-group availability

This was investigated by examining amino, carboxy and thiol group availability by using the techniques described under 'Methods'. It can be seen in Table 1 that the number of available thiol and carboxy groups is similar in fibronectin extracted from the culture medium of LA31 NRK normal and transformed cells. As regards amino groups, there is a 50% increase in their availability in the fibronectin purified from the medium of LA31 NRK cells at 33°C compared with that in the fibronectin from LA31 NRK cells at 39°C.

Before ascribing any significance to this dif-

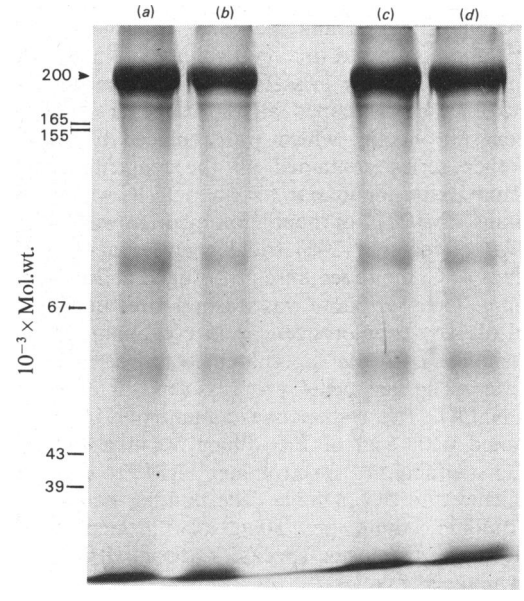

Fig. 1. *Polyacrylamide-gel electrophoresis of fibronectin extracted from the culture medium of normal LA31 NRK cells at 39°C (a) or transformed LA31 NRK cells at 33°C (b), B77 NRK cells at 39°C (c) and B77 NRK cells at 33°C (d)*

The position of standards [RNA polymerase (mol.wt. α subunit 39000, β subunit 155000, β' subunit 165000), bovine serum albumin (mol.wt. 67000) and ovalbumin (mol.wt. 43000)] is indicated on the photograph.

Table 1. *Sialic acid content and quantitative determination of the number of available amino, thiol and carboxy groups on fibronectin from normal and transformed cells*

All determinations were performed in duplicate and as described in the Experimental section. Duplicate values did not vary more than 8%. Values reported are means of two experiments.

		Content as end groups (nmol/mg of fibronectin)			
	Cell type	Amino (Lys)	Thiol (Cys)	COOH (Glu and Asp)	Sialic acid (nmol/mg of fibronectin)
NRK LA31	Transformed (33°C)	460	33	6.3	73
	Normal (39°C)	307	35	5.7	71
NRK B77	Transformed (33°C)	260	63	3.7	136
	Transformed (39°C)	286	63	3.1	148

ference in amino group availability, we tried to determine whether the temperature change itself had any influence on the results. Accordingly, end-group availability was investigated on fibronectin extracted from the medium of NRK cells transformed by the wild-type avian sarcoma virus (NRK-B77) and subjected to the same temperature shift. From the results presented also in Table 1, it is apparent that there are no significant changes in thiol, amino and carboxy group availability between the fibronectins from the media of NRK-B77 cells incubated at either 33°C or 39°C. However, in absolute terms, fibronectin from the medium of NRK-B77 cells, compared with that from the medium of LA31 NRK cells, shows about a 2-fold increase in thiol group availability and sialic acid content but a corresponding 2-fold decrease in amino and carboxy group availability. There is no immediate explanation for this difference.

The changes in amino group availability between fibronectin from the media of LA31 NRK cells at 33°C and 39°C prompted us to determine whether the increased amino group availability was due to a decrease in isopeptide bond formation (between the ε-amino groups of lysine and the amide groups of glutamine), to a greater polyamine content or to changes in secondary structure.

Polyamine binding capacity of fibronectin extracted from culture media

Spermidine was incorporated covalently into fibronectin by the action of transglutaminase as described under 'Methods'. Kinetic evidence for the transglutaminase reaction developed by Gross *et al.* (1977) has demonstrated quite clearly that the amine enters the reaction sequence only after the formation of an acyl-enzyme intermediate between the acceptor protein (which contributes the acyl portion) and the enzyme itself. On this basis, the affinity for a given amine is a function of the structure of the acyl portion of the first substrate, which in this case is fibronectin.

Classically, the K_m of the enzyme for fibronectin should have been determined by varying the fibronectin concentration in the presence of an excess of amine. However, this was not possible experimentally since reproducible kinetic data could only be obtained with dilute solutions of fibronectin around 120 μg/ml. The tendency of fibronectin to polymerize in the presence of polyamines had previously been reported by Vuento *et al.* (1980). These authors showed that with spermidine or putrescine (between 0.1 and 1 mM) at 37°C no aggregation of fibronectin (400 μg/ml) occurred, whereas aggregation took place with spermine (between 0.5 and 1 mM). For this reason, the apparent $V_{max.}$ and the apparent K_m of the transglutaminase-mediated insertion of polyamines into fibronectins were determined with spermidine. The Lineweaver–Burk representation of the results showed (Fig. 2) that two types of kinetics can be detected, one for fibronectin from LA31 NRK cells at 33°C, indicating kinetics typical of inhibition by high substrate concentration, and a second for fibronectin from LA31 NRK cells at 39°C, represented by a straight line. In the second case, the apparent K_m for spermidine was 102 μM, whereas the apparent $V_{max.}$ value was 77 pmol of spermidine/50 μg of fibronectin.

The same experimental approach with fibronectin extracted from the medium of cells transformed by the wild-type virus B77 showed (Fig. 3) that at both temperatures only the kinetics typical of substrate inhibition was observed. Possible reasons for these curved kinetic data are discussed below. However, from these data it is clear that differences in polyamine acceptor capacity of fibronectins from normal and transformed cells are not due to the temperature shift in itself but rather to the altered binding capacity of fibronectin from the transformed cells.

Polyamine content of acid-hydrolysed fibronectins

To determine whether there were differences in the polyamine content of the fibronectin from the culture medium of normal and transformed cells, purified fibronectin was acid-hydrolysed and its polyamine content determined as described in the Experimental section. Only one radioactive peak with the same retention time as authentic spermine was detected

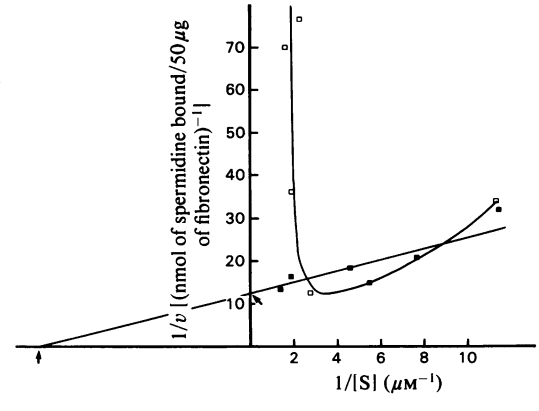

Fig. 2. *Transglutaminase-mediated insertion of spermidine into fibronectin from culture media of normal cells (LA31 NRK at 39°C)* (■) *and transformed cells (LA31 NRK at 33°C)* (□)

The arrows indicate $1/V_{max.}$ (apparent) and $1/K_m$ (apparent). Each point represents the average of two determinations carried out in two different experiments.

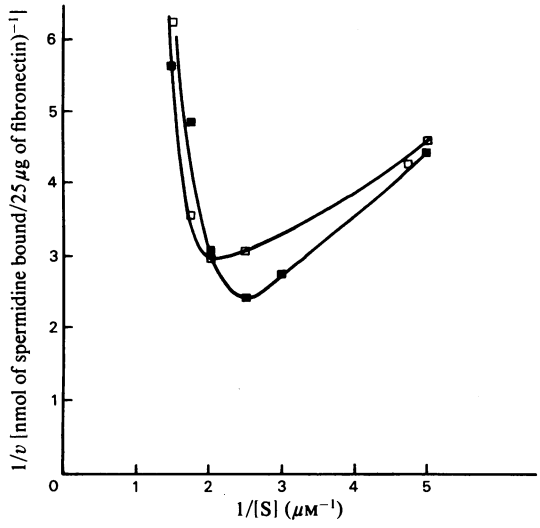

Fig. 3. *Transglutaminase-mediated insertion of spermidine into fibronectin extracted from culture medium of transformed cells*

■, B77 NRK cells at 39°C; □, B77 NRK cells at 33°C. Each point represents the average of two determinations carried out on two different experiments.

for fibronectin from LA31 NRK cells at 33°C or at 39°C. It showed that fibronectin from LA31 NRK cells at 33°C contained 0.037 pmol of spermine/mg and that from LA31 NRK cells at 39°C 0.039 pmol of spermine/mg. There is no significant difference. As the difference in reactivity of the two fibronectins was apparently not due to differences in their endogenous spermine content, possible changes in secondary structure were examined immunochemically.

This was performed quantitatively by measuring the reactivity of each of the fibronectins with two different antisera; one of which was directed against human fibronectin, the other against spermine.

Reaction with antifibronectin antiserum

It has previously been shown by Kuusela *et al.* (1976) that an anti-(human fibronectin) antiserum precipitates other fibronectins of mammalian origin. We therefore used such an anti-(human fibronectin) antiserum to examine the common antigens of fibronectins from normal and transformed LA31 NRK cells. It is apparent from Fig. 4 that the two fibronectins react differently: there are more antigenic sites available on fibronectin prepared from the culture medium of normal than on that prepared from the medium of transformed cells.

Since the antigenic sites with which the antibodies react were not known, we examined the availability of one type of constituent that could be part of an antigenic site and that has been previously identified

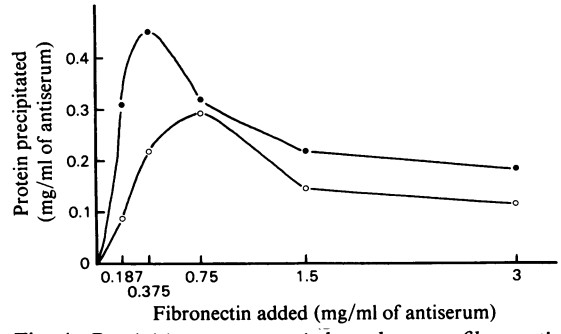

Fig. 4. *Precipitin curves carried out between fibronectin extracted from the culture medium of normal LA31 NRK cells at 39°C* (●) *or transformed LA31 NRK cells at 33°C* (○) *with anti-fibronectin antiserum*

The amount of protein precipitated was determined by the technique of Lowry *et al.* (1951).

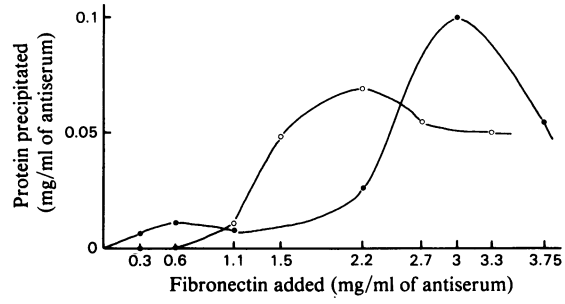

Fig. 5. *Precipitin curves carried out between fibronectin extracted from the culture medium of normal LA31 NRK cells at 39°C* (●) *or transformed LA31 NRK cells at 33°C* (○) *with anti-polyamine antiserum*

The amount of protein precipitated was determined by the technique of Lowry *et al.* (1951).

on the fibronectin molecule, namely polyamines (Roch *et al.*, 1980).

Reaction with anti-polyamine antiserum

The results shown in Fig. 5 indicate that maximum precipitation is reached with 2.2 mg of fibronectin isolated from the medium of transformed cells, whereas it requires 3 mg of fibronectin isolated from the medium of normal cells to attain maximum precipitation. However, it should be noted that the amount of proteins precipitated is greater with the fibronectin from normal than with that from transformed cells. One factor that may contribute to this latter observation is the heterogeneity of anti-polyamine antiserum, which has been shown to contain distinct populations of antibodies to spermine, spermidine and putrescine in decreasing order of magnitude (Jonard *et al.*, 1967). One possible interpretation of this precipitin curve is that there are more polyamine sites available to the antibodies on

fibronectin prepared from the culture medium of transformed cells than on that prepared from the culture medium of normal cells.

Discussion

The results presented here provide evidence for a 50% increase in the availability of amino groups on the fibronectin isolated from the culture medium of transformed rat kidney cells compared with that on medium fibronectin from normal cells.

The aforementioned difference was due neither to differential proteolytic cleavage of the molecule (Fig. 1) nor to the temperature shift itself, as fibronectins from cells transformed by the wild-type virus B77 at both temperatures exhibit the same characteristics.

Furthermore, amino acid analysis of these two fibronectins (i.e. at 33 and 39°C) showed no differences, except small increases of the order of 20–30% in lysine, histidine and proline in the fibronectin from cells at 33°C compared with that from cells at 39°C and a 60% higher glycine content in fibronectin from cells at 39°C compared with those at 33°C. The small increase in lysine could therefore contribute to the greater amino group availability, but the significance of the increase in glycine residues in fibronectin from cells at 39°C is not clear. Nevertheless, rat fibronectin from cells at both 33 and 39°C does show another unusual characteristic in its aspartate and glutamate content compared with that of human plasma fibronectin [962 nmol/mg in the former compared with 1900 nmol/mg in the latter (Mosesson *et al.*, 1975)]. This result together with those in Table 1 suggest either that the degree of amidation of rat kidney fibronectin is greater than 99% or that the majority of free carboxy groups are buried inside the molecule.

Unfortunately, the destruction of half-cystine residues on protein hydrolysis, in spite of the presence of a thiol-protecting reagent, did not permit us to obtain additional evidence for the large number of free thiol groups detected by the Ellman method.

Differences in the number of isopeptide bonds (Folk *et al.*, 1980) could also have contributed to changes in the availability of amino groups. However, their direct measurement would have required quantities of fibronectin much greater than those that can be prepared normally from 1 litre of culture medium.

As regards their spermidine acceptor capacity, precise values for K_m and $V_{max.}$ could be obtained only with the fibronectin from cells at 39°C. With fibronectin from cells at 33°C or from cells transformed by the wild-type virus, a curve typical of inhibition by excess substrate was obtained. This was seen neither with the fibronectin from the media of normal cells nor with casein as acceptor proteins. The curve cannot be due either to the presence of non-cellular fibronectin from the horse serum in the medium, since this amount was essentially the same at both 33 and 39°C, or to the type of amine used, as comparable results were obtained with putrescine (results not shown). It seems highly unlikely then that this inhibition is due to an effect of the polyamine on the transglutaminase itself. If the non-linear kinetics were due to an effect of the polyamine on the acceptor protein, then a possible explanation for the kinetic data might be the following. (1) The acceptor protein from the medium of transformed cells contains more polyamines in isopeptide linkage. This would be compatible with the increase found in amino group determination. (2) The interaction of polyamines with fibronectin from transformed cells brought about changes in the secondary structure of the fibronectin. A consequence of such a change would be a decrease in the availability of glutamine-binding sites. Hence less acyl-enzyme intermediates would be formed. As a result, less polyamines would be bound as the polyamine concentration increases.

It was possible to exclude experimentally the first of these two hypotheses.

The results showed that bound polyamines are present in only minute amounts (0.04 pmol/mg of protein) and further that there is no significant difference in the spermine content of the fibronectins prepared from the media of normal and transformed cells. The small amounts of polyamines found in fibronectins are in agreement with the results of Folk *et al.* (1980), who found that only a very small proportion of polyamines given to stimulated lymphocytes is recovered as protein-bound polyamines.

In the absence of any direct experimental verification of the second hypothesis, the results of our immunochemical experiments with both anti-fibronectin and anti-polyamine antisera suggest that: (*a*) the antigenic sites common to other mammalian fibronectins are less accessible on the surface of fibronectin from cells at 33°C compared with those on fibronectin from cells at 39°C (Fig. 4); (*b*) polyamine-binding sites are more accessible on the fibronectin from cells at 33°C than on that from cells at 39°C (Fig. 5).

However, with both antisera other interpretations could have been that differences exist in the actual number of antigenic sites or in their accessibility to the antibodies due to alterations in secondary structure. Our results do not permit us to choose between these two alternatives.

One mechanism that could bring about changes in the secondary structure and hence antigenicity of fibronectin from transformed cells is increased sulphation (Wilson *et al.*, 1981) and/or phos-

phorylation (Teng & Rifkin, 1979). However, the generality of this latter observation is open to question in view of the results of Ali & Hunter (1981) showing decreased phosphorylation. Nevertheless one consequence of this altered structure could be a modification of the cell-binding capacity of fibronectin of transformed cells. This may be responsible for its diminished adherence to the surface of such cells (Yamada *et al.*, 1981).

Another consequence of this altered structure is that the fibronectin molecule could acquire a net surface charge, which would account for the differences in isoelectric point between fibronectins from human germ cell tumours and plasma (Ruoslahti *et al.*, 1981*b*). An antigenicity not associated with the native molecule could also result from structural changes. Were this the case *in vivo*, it would provide one explanation for the immunogenic stimulus responsible for eliciting natural antibodies to polyamines that have been detected in man (Roch *et al.*, 1978, 1981) and in experimental animals (Bartos *et al.*, 1980; Furuichi *et al.*, 1980).

We thank Dr. Louis Gazzolo for stimulating discussion, Dr. Madeleine Duc Dodon for the sialic acid determination, Professor Jean Gras for performing the amino acid analyses, Dr. Victor Villanueva and Dr. Joseph Huppert for helpful discussion and Miss Aline Mary for efficient secretarial help. This investigation received financial support from the Fédération Nationale des Centres de Lutte contre le Cancer 'Vaincre le Cancer' (France).

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