Hamster cell fibronectin is a glycoprotein consisting of two 230,000-dalton subunits in a disulfide-bonded dimer. The molecule is composed of domains which can be separated by partial proteolytic cleavage. The carbohydrates, disulfide bonds, and a single free sulfhydryl group per chain are distributed nonuniformly among these regions. All the interchain disulfides are within 10,000 daltons of the end of the molecule and are removed by mild proteolysis which also generates 200,000- and 25,000-dalton fragments which do not contain interchain disulfides. The 200,000-dalton fragment contains all or most of the carbohydrate side chains, and the free sulfhydryl group, but is relatively poor in cystine. The 25,000-dalton fragment is carbohydrate-free and cystine-rich but has no free sulfhydryl groups. There is heterogeneity in carbohydrate content among the monomeric chains of intact fibronectin and the 200,000-dalton fragments. The gelatin binding site of fibronectin is in the 200,000 fragment. Intact disulfide bonds are required for binding of fibronectin to cells and to gelatin and blockage of the free sulfhydryl groups prevents binding of fibronectin to cells, suggesting that intermolecular disulfide bonding may be important.

Fibronectin is a major surface glycoprotein of fibroblasts and some other cell types and is greatly reduced in amount after oncogenic transformation (1–3). It comprises part of the cell surface coat of the cells (4, 5) and exists in a fibrillar network between cells and substrata (6), in regions of cell-cell contact (6, 7), and in dense cultures as an elaborate network covering the cells (6, 8, 9). It appears to be involved in adhesion of cells to substrata and its presence affects a variety of phenotypic properties of cells including adhesion, spreading, morphology, overlapping, and alignment of cells (10, 11). Fibronectin has a high affinity for collagen, a property which could be involved in some of its biological functions (12–15). Addition of fibronectin to transformed cells causes reappearance of microfilament bundles within the cells (11, 16) and promotes cell migration (17). In normal cells the distribution of fibronectin correlates with that of actin (18). These properties indicate that fibronectin is an important constituent of the cell surface.

In this research we have investigated the overall structure of fibronectin and the way this relates to some of its biological functions. Fibronectin is a disulfide-bonded dimer. After reduction of the disulfide bonds with dithiothreitol, fibronectin is unable to initiate any of the morphological changes usually observed upon its readdition to transformed cells (19). Reduction of disulfide bonds also leads to release of fibronectin from normal cells, and it is known that cell surface fibronectin is involved in high molecular weight aggregates held together by disulfide bonds (20, 21). We have therefore paid particular attention to the presence and role of disulfide bonds and sulfhydryl groups which appear important for the expression of the biological functions of fibronectin.
elution of fibronectin which was 95% pure. For a few experiments
\(^{[3]H}\)glucosamine-labeled fibronectin was purified only by precipita-
tion with 30% ammonium sulfate. Fibronectin was also purified from
secondary chick embryo fibroblasts as described by Yamada et al
(10). It should be noted that fibronectin isolated from cell surface
and culture medium have been shown to be indistinguishable in size and
biological activity (23).

**Amino Acid Composition**—For amino acid analysis, fibronectin
from NIL8 culture medium was purified on gelatin-Sepharose and
then further purified by chromatography on Sepharose 4B. Fractions
were checked for purity on SDS-polyacrylamide gels. Prior to acid
hydrolysis, half-cystine residues were modified one of the following
ways: (a) conversion of half-cystines to cysteic acid. The Sepharose 4B
column was run in 4 M urea in CAPS buffer. Samples were checked
for purity on gels, dialed into 0.1 M acetic acid, and oxidized with
performic acid (24). (b) Conversion of half-cystines to S-(4-pyri-
dyldethyl) cysteine (Cys(PyrEt)) (Ref. 25). The Sepharose 4B column
was run in 8 M urea, pH 7.5, 0.07 M Tris buffer. To the samples
mercaptoethanol was added (about 100 molar excess over total disul-
fide) and the mixture was stirred overnight at room temperature
(under nitrogen). 4-Vinylpyridine was added (1:1 ratio to total sul-
fhydryl groups) and the mixture stirred for 2 h. The pH was lowered
to 3.5 with glacial acetic acid and the sample was dialyzed extensively
against 0.1 M acetic acid. (c) Conversion of half-cystines to carboxy-
myristoylated fibronectin (Cys(Cm)-containing). After the Sepharose 4B
column step, samples were dialed into 0.1 M acetic acid and freeze-dried. The dry
sample was dissolved in 6 M guanidine HCl in 0.2 M Tris, pH 8, and
reduced with 10 mM dithiothreitol for 1 h at 37°C. Recrystallized
iodoacetic acid was added (30 mM) and the mixture kept in the dark
for 1 h at room temperature. Reaction was stopped with a small drop
of mercaptoethanol. The sample was dialed extensively against 0.1 M
acetic acid.

All samples were repeatedly lyophilized and were then hydrolyzed
in 6 M HCl at 110°C for 24 h (Cys(PyrEt)- and Cys(Cm)-containing
samples also contained 0.2% mercaptoethanol) and the hydrolysates
were analyzed on an amino acid analyzer in comparison with appro-
priate markers.

\[^{[3]H}\]Iodoacetic Acid Labeling—Fibronectin was reduced (10 mM
dithiothreitol, 37°C, 90 min) or not, and then dialed into 8 M urea
dissolved in NaCl/Pi, 0.1 M Tris, pH 8.0, or CAPS buffer, pH 11.
Fibronectin at 280 to 500 μg/ml was reacted with 200 μCi/ml of
\[^{[3]H}\]iodoacetic acid (New England Nuclear, 200.8 μCi/mmol) for 90
min at 37°C in the dark. Aliquots were taken for precipitation by
trichloroacetic acid and for analysis on SDS-polyacrylamide gels.

**Blocking of Free Sulfhydryl Groups with N-Ethylmaleimide**
(MalNEt)—After elution of fibronectin from gelatin-Sepharose in 4
M urea in CAPS buffer, solid MalNEt was added to a final concentra-
tion of 10 mM and incubated for 30 min at room temperature. Samples
were then dialyzed against CAPS buffer before being added to the
cells.

The efficiency of blocking of the sulfhydryl groups was checked on
an activated thiol-Sepharose 4B column (see below).

**Detection of Free Sulfhydryl Groups by Covalent Chromatogra-
phy**—A 2-ml column of activated thiol-Sepharose 4B (Pharmacia)
was loaded with 300 μl of freshly purified \[^{[3]S}\]methionine-labeled
fibronectin (0.1 M Tris, pH 8, buffer, 8 M urea, 1 M EDTA). After 3
h incubation, the column was washed with the same buffer and then
with this buffer containing 1 M NaCl. Disulfide bonds were then
broken with 10 mM dithiothreitol in the same buffer.

**Quantitation of Free Sulfhydryl Groups**—During the usual puri-
ification steps of fibronectin all buffers were degassed and flushed with
nitrogen. To the column was added (in the presence of nitrogen)
dithiobis(2-nitrobenzoic acid) (NBzS₂), and the column was eluted
with 1 mM NBzS in 8 M urea, 0.1 M Tris/sulfate buffer, pH 8.0. The
euate was dialyzed extensively against 4 M urea 0.1 M Tris/sulfate,
pH 8.0. Then the protein concentration was determined by the method
of Lowry using bovine serum albumin as standard. The bound
thionitrobenzoic acid was released by addition of dithiothreitol to the
column at a final concentration of 10 mM. The increase of optical
density was read at 412 nm (ε₄₁₂ for reduced NBzS₂ = 1.36 × 10³).

**SDS-Polyacrylamide Gels**—Analysis of samples was on slab gels
in the buffers described by Laemmli (26). Samples were boiled in a
2% sodium dodecyl sulfate (SDS) either with or without 0.1 M
dithiothreitol. Molecular weights were calibrated using markers; fibro-
nectin (230,000), ovalbumin (45,000), myosin (200,000), lysozyme
(14,000); β-lactoglobulin (180,000); β-galactosidase (130,000); phos-
phorylase A (94,000); bovine serum albumin (66,000), catalase (60,000);
and Sinedin virion proteins (53,000 and 30,000) which were a generous gift of Dr. D. Wirth, Massachusetts
Institute of Technology. All molecular weights of fragments are
approximate. Radioactivity was detected by autoradiography of dried
geles (\[^{[3]H}\]) or by fluorography after impregnation with 2,5-diphenyl-
oxazole (27) for \[^{[3]}\) and \[^{[35]}\)S. Stained gels and autoradiograms were
scanned on a Zeineh soft laser densitometer. For quantitation, gels
were exposed for varying lengths of time to preflashed Kodak x-ray
film. Absorbance as a function of exposure time indicated the linearity
of the response of the film.

**Double Label Experiments**—To check relative distributions of
different constituents among tryptic fragments, fibronectin was puri-
ified from cells labeled in parallel with \[^{[3]H}\]glucosamine and either a
[^{[3]}]C-amino-acid mixture or \[^{[35]}\]S-cysteine. The purified fibronectin
were mixed to give a \[^{[3]H}\]/\[^{[35]}\]S ratio of about 10 and the double
labeled mixture was dialyzed and digested. Samples were run on
5% SDS-polyacrylamide gels which were sliced into 1-mm slices.
The slices were dissolved in 200 μl of H₂O₂ in capped vials at 80°C for
20 h and counted using Triton-toluene scintillant. Calculations of
distribution of glucosamine and cystine were as follows. It was as-
sumed that the \[^{[3]}\]C-amino-acid mixture gave uniform labeling. If
recovery of a 200-kd fragment were 100%, then recovery of \[^{[3]}\]C-amino-
acids should be 87% (20/230). The recovery of \[^{[3]}\]Hglucosamin in this
fragment was therefore corrected using this value and the actual
recovery of \[^{[3]}\]S. The calculated recovery of \[^{[3]}\]Hglucosamin was then
used to correct the recovery of \[^{[3]}\]S-cystine.

**RESULTS**

**Location of Interchain Disulfide Bonds**—Fibronectin on the
cell surface is very susceptible to proteolysis (1, 22, 28–
30). To study the fragments formed by tryptic digestion, idinated dishes of NIL8 cells were treated with trypsin at
varying concentrations and times. Reaction was stopped by
soybean trypsin inhibitor. Samples of the liquid phase and of
the cell lysates were run on SDS polyacrylamide gels with
or without reduction (Fig. 1). In both cases strong bands corre-
sponding to polypeptides of molecular weight around 200,000 (co-
migrating with myosin) appeared on the cells and were
released into the digests. Precipitation with antibody to fibro-
nectin showed that these bands have antigenic determinants
different from (not shown). This means that cell surface fibro-
nectin has an asymmetric distribution of interchain disulfide
bonds. A stretch of 200 kD in each half of the disulfide-
bridged dimer possesses no disulfide bridges binding it coval-
ently to the other polypeptide chain. This 200-kd fragment
therefore behaves in the same way if run reduced or non-
reduced.

To test whether the resistance of the 200-kd fragment to
proteolysis was intrinsic or caused by its associations on the
cell surface, we studied fibronectin in solution. Fibronectin
purified from chick embryo fibroblasts or from NIL cells was
also rapidly cleaved by trypsin (1 μg/ml) to give 200-kd
fragments which were then relatively resistant to further
cleavages. The 200-kd fragments were again released with or
without subsequent reduction (see below). This 200-kd polypep-
tide was also obtained whether or not the protein was
previously reduced with dithiothreitol (not shown).

At short digestion times, fragments of intermediate sizes
215,000 and 220,000 daltons could be observed (Fig. 2). Both
these fragments migrated at the same positions reduced or
nonreduced. An additional fragment of 240,000 appeared only
on the nonreduced half of the gel. These results indicated
several tryptic cleavage sites in the susceptible 200,000-dalton
region and indicate that the interchain disulfide bonds are
within 10 kD of the end of the intact 230-kd molecule.

**Distribution of Carbohydrate and Half-cystine**—In experi-
ments with \[^{[3]}\]Hglucosamine-labeled fibronectin prepared by
ammonium sulfate precipitation, we observed that 90 to 100%
of the carbohydrate label was conserved in the 200-kd frag-
ment. Since this preparation was not completely pure, the
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FIG. 1. Tryptic digestion of iodinated NIL8 cells. Cells were treated for indicated time period (minutes) with 1 µg/ml of trypsin. Samples from cell lysates (A) and the supernatants (B) were run on 5% SDS-polyacrylamide gels reduced (R) and nonreduced (N). The appearance of 200,000-dalton fragments (arrows) can be observed on both the reduced and nonreduced gels.

experiments were repeated with fibronectin purified on gelatin-Sepharose. This procedure involves elution with 4 M urea and stoichiometric recovery of the 200-kd fragment after trypsin treatment is no longer observed. This may suggest some denaturation by the urea. To allow calculation of the relative distributions of carbohydrate and half-cystine in the 200-kd fragment, fibronectin labeled with both [3H]glucosamine and either [14C]-amino-acid mixture or [35S]cystine was purified and treated with trypsin (see “Experimental Procedures”). Results are shown in Fig. 3.

Several conclusions can be drawn from these results. First, the carbohydrate/amino acid ratio ([3H]/[35S] or [3H]/[14C]) is consistently higher at the slower migrating portion of the fibronectin band (Fig. 3, A and C). In high resolution gels we are sometimes able to resolve two bands of fibronectin after reduction. The results in Fig. 3 suggest that they may differ

* Unpublished data.

in their carbohydrate content. After cleavage with trypsin, the asymmetry of carbohydrate content persists (Fig. 3, B and D), suggesting that the different bands in the 200-kd region also differ in number of length or carbohydrate side chains. In experiments comparing [3H]glucosamine with [14C]-amino-acid mixture, recovery of the two was similar whereas recovery of [35S]cystine in the 200-kd fragment was markedly less (Fig. 3D). Using the recovery of [14C]-amino-acids as comparison, one can calculate that 66 to 80% of the carbohydrates ([3H]glucosamine) present in intact fibronectin is recovered in the 200-kd fragments. Using this value and the relative recoveries of [3H]glucosamine and [35S]cystine, one can calculate that 34 to 42% of the half-cystines of intact fibronectin are located in the 200-kd fragment. These results are based on 66 to 80% recovery of carbohydrates in the 200-kd fragment. Since earlier results obtained with fibronectin not treated with urea have suggested higher recoveries in this fragment, these calculations of half-cystine content of the 200-kd fragment possibly represent a slight underestimate. If one assumes that 100% of the carbohydrate is in the 200-kd fragment, the observed recovery of [35S]cystine in this fragment would predict that 50% of the half-cystines are in the 200-kd fragment. Combining the two sets of data, it is clear that only 34 to 50% of the half-cystines are present in the 200-kd fragment. This leaves 50 to 66% of the half-cystines in the 30-kd region removed by proteolysis.

Amino acid analyses of fibronectin, carried out according to several different protocols with particular attention to half-cystine residues, indicate 40 to 48 half-cystine residues per chain (Table I). Of these, 14 to 24 are in the 200-kd fragment and 20 to 32 are in the 30-kd region removed by proteolysis.

Analysis of tryptic digests of fibronectin on 10% SDS-polyacrylamide gels showed release of the 200-kd fragment and simultaneous appearance of a 25-kd fragment. This 25-kd peptide migrated faster (apparent molecular weight, 19 kd) on the nonreduced half of the gel (Fig. 4A) suggesting the presence of intra- but not interchain disulfide bridge(s). No disulfide-bonded fragment corresponding with the piece which covalently binds the monomers of fibronectin was detected on these gels. An unlikely possibility is that the 25-kd fragment actually exists as a dimer which migrates with an apparent molecular weight of 19 kd. However, since this would require
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3H

wm

6OOC I-

I-

I-

I-

I-

600 -6

400 -4

200 -2

I 1 1

8 20 min

300

100

15 20 25 30 35 40

FIG. 3. Digestion of double labeled fibronectin. M, [3H]glucosamine counts; O—O, 14C or ^35S counts (from 14C-amino-acid mixture in A and B; from [35S]cystine in C and D). △—△, ratio H/14C or H/^35S. A, [3H]glucosamine- and 14C-amino-acid-labeled fibronectin. Note asymmetry of H/14C ratio. B, the same sample after 20 min digestion with 1 µg/ml of trypsin. The overall ratio H/^35S increased in comparison with the intact fibronectin. This suggests that [35S]cystine counts were removed preferentially (see text).

TABLE I

Amino acid composition of hamster cell fibronectin

Each column represents the average of two determinations on separate preparations of fibronectin. Half-cystine content was determined as cysteic acid, pyridyl ethyl cysteine, or carboxymethylcysteine, under “Experimental Procedures.”

| No. of residues/230,000 daltons | Average |
|-------------------------------|---------|
|                               | Residues | Mole % |
| Cysteic acid                  | 47       | 40     | 44     | 2.3    |
| Cys(PyrEt)                    |          |        |        |        |
| Cys(Cm)                       | 45       |        |
| Aspartic acid                 | 176      | 162    | 154    | 164    | 8.8    |
| Methionine                    | 20       | 33     | 25     | 26     | 1.4    |
| Threonine                     | 176      | 135    | 144    | 152    | 8.1    |
| Serine                        | 133      | 109    | 125    | 122    | 6.5    |
| Glutamic acid                 | 226      | 225    | 225    | 225    | 12.0   |
| Proline                       | 156      | 167    | 190    | 171    | 9.1    |
| Glycine                       | 221      | 276    | 259    | 252    | 13.5   |
| Alanine                       | 100      | 111    | 117    | 109    | 5.8    |
| Valine                        | 127      | 113    | 124    | 121    | 6.5    |
| Isoleucine                    | 83       | 78     | 83     | 81     | 4.3    |
| Leucine                       | 101      | 98     | 107    | 102    | 5.4    |
| Tyrosine                      | 47       | 63     | 45     | 52     | 2.8    |
| Phenylalanine                 | 47       | 46     | 50     | 46     | 2.5    |
| Histidine                     | 36       | 46     | 33     | 38     | 2.0    |
| Lysine                        | 62       | 70     | 66     | 66     | 3.5    |
| Arginine                      | 99       | 99     | 99     | 99     | 5.3    |

To verify the presence of free sulfhydryl groups on fibronectin we have used an activated thiol-Sepharose 4B column which covalently binds proteins containing free sulfhydryl groups. Fibronectin binds to this column and can be eluted with 10 mM dithiothreitol (Fig. 5). Pretreatment of the column with NBsS2 did not prevent binding of fibronectin while pretreatment of fibronectin with NBsS2 blocked its binding to the column (Fig. 5). When the partial tryptic digest fragments were loaded on this activated thiol-Sepharose column, the 200-kd fragment bound while the 25-kd fragment was in the flow through (Fig. 6).
**Fig. 4.** Large and small fragments derived from fibronectin. 10% SDS-polyacrylamide gel showing smaller fragments resulting from trypsin digestion (1 µg/ml). *Numbers* indicate time of treatment in minutes. *A,* fragments from [35S]cysteine-labeled fibronectin. Two new bands appear; a strongly labeled 25-kd fragment migrating faster nonreduced (N) than reduced (R), plus a faint band of about 40 kd; *B,* [3H]glucosamine-labeled fibronectin digested and run reduced. Note that the 25-kd fragment was not labeled with glucosamine whereas the 200-kd (and faintly the 40-kd) fragment was labeled.

**Fig. 5.** Detection of sulphydryl groups in fibronectin. [35S]cysteine-labeled fibronectin and fibronectin reacted with NBsS$_2$ (DTNB) were loaded on two activated thiol-Sepharose columns. Columns were first washed with buffer, then with buffer and 1 M NaCl, and finally eluted with dithiothreitol (DTT). Practically all the fibronectin sticks to the control column. By contrast, the sample whose free sulphydryl groups were blocked (NBsS$_2$) came off in the flow-through.

**Fig. 6.** Location of sulphydryl groups in fragments. Tryptic digest of [35S]methionine-labeled fibronectin was run on an activated thiol-Sepharose 4B column. Samples from the column were run on 10% SDS-polyacrylamide gel. Note that the 25-kd fragment does not bind and comes in the flow-through whereas the 200-kd fragment and the 40-kd fragment bind and elute with dithiothreitol (DTT).

**Fig. 7.** Quantitation of sulphydryl groups of fibronectin. Fibronectin bound to gelatin-Sepharose was washed with buffer containing NBsS$_2$ and eluted with 8 M urea, pH 8.0, containing NBsS$_2$. The eluted fractions were dialyzed and their protein content (○---○) determined. Bound thionitrobenzoic acid was released with dithiothreitol and determined by optical density at 412 nm (●---●). The numbers indicate the calculated sulphydryl content per 230,000 daltons of protein.
To determine the number of free sulphydryl groups per fibronectin molecule, we reacted hamster fibronectin with 1 mM NBzS₂ in 8 M urea during purification on gelatin-Sepharose (see “Experimental Procedures”). Excess NBzS₂ was then dialyzed away and the protein concentration and the increase in absorption at 412 nm after reduction of the NBzS₂-reacted fibronectin were measured. Fig. 7 shows the results of a typical experiment. Calculation showed the presence of one free sulphydryl group per 230,000 daltons of protein. Lower values (0.35 to 0.51) were obtained after reaction with NBzS₂ in 4 M urea, suggesting incomplete reaction of the sulphydryl groups at this lower urea concentration.

In summary, the activated thiol-Sepharose quantitatively binds both intact fibronectin and the 200-kd fragment indicating the presence of free sulphydryl groups in each of these molecules. Quantitation shows one (and not more than two) sulphydryl groups per polypeptide chain of fibronectin. This free sulphydryl is located in the 200-kd region.

Biological Relevance of Sulphydryl Groups and Disulfides—It was previously shown that intact disulfide bridges in fibronectin are necessary for its biological activity when added to transformed cells, and for its retention at the surfaces of normal cells (19). One interaction possibly involved in biological activities of fibronectin is its interaction with collagen (12–14). As fibronectin binds to gelatin (denatured collagen), we have used this property to investigate the requirement for intact disulfide bonds for gelatin binding. Aliquots of culture medium containing [³⁵S]cystine-labeled fibronectin were run on gelatin-Sepharose columns with or without prior reduction with 50 mM dithiothreitol. Bound fibronectin was eluted with 4 M urea. In the nonreduced sample, all the fibronectin was bound; after reduction, practically none bound (Fig. 8). Hence the disulfide bonds are required for efficient binding of fibronectin to gelatin and, presumably, to collagen. The gelatin binding site is situated in the 200-kd fragment which can be purified on a gelatin-Sepharose column (not shown).

NIL cells will bind fibronectin added to the culture medium (10, 11, 23). We have purified [⁴⁰S]methionine-labeled fibronectin, blocked one part of the sample with N-ethylmaleimide and added it to NIL8 cells. Samples from media and cell lysate were then counted. There was a 10-fold decrease in the binding of fibronectin treated with MalNEt in comparison with un-
with the binding of fibronectin to the cell surface.

2 and 3 and the 25-kd fragment from Cuts 1 and 3 in the absence of blocking the free sulfhydryl groups on fibronectin interferes alkylation with iodoacetic acid. These results indicate that treated samples (Fig. 9). A similar result was obtained after treatment interferes with binding of fibronectin to cells.

MalNEt treatment interferes with binding of fibronectin to cells. [35S]Methionine-labeled fibronectin, treated or untreated with N-ethyl maleimide, was added in 2 ml of medium to 3-cm dishes of NIH 3T3 cells, which were lysed in 220 μl. A, top graph: increasing numbers of counts were added; incubation was for 25 hours; B, bottom graph: equal numbers of counts were incubated for varying times. Note that MalNEt treatment interferes with binding of fibronectin to cells.

FIG. 10. Models for the structure of hamster cell fibronectin. The diagrams depict the three identified domains: a very short (~10 kD) region containing the interchain disulfide bonds, a 25-kd region rich in intrachain disulfides and free of carbohydrate, and a 200-kd region containing all or most of the carbohydrate, the free sulfhydryl and relatively few intrachain disulfides. Two alternate sequences for the arrangement of these domains are shown but are tentative (see text). Not all carbohydrate side chains are shown; nor are all the (>40) sulfhydryl bonds. The location of the free sulfhydryl groups is arbitrary. Three proteolytic cleavage sites are required to generate the fragments observed (see Figs. 2 and 4). The 240-kd piece would arise from Cuts 3 in only one chain, the 215-kd and 220-kd fragments from Cuts 2 and 3 and the 25-kd fragment from Cuts 1 and 3 in the absence of Cut 2. Location of the rare 40-kd fragment is unknown but it contains carbohydrate and a free sulfhydryl, so presumably arises from the 200-kd fragment by further cleavage.

treated samples (Fig. 9). A similar result was obtained after alkylation with iodoacetic acid. These results indicate that blocking the free sulfhydryl groups on fibronectin interferes with the binding of fibronectin to the cell surface.

DISCUSSION

The results described above are incorporated into models for the structure of hamster cell fibronectin which are depicted in Fig. 10. Three distinct regions within each chain of the dimer are identified and can be separated from each other by partial proteolysis. There are: 1) a 200-kd region containing most or all of the carbohydrate and less than half the cysteine residues but all the cysteine. 2) a 25-kd region which is carbohydrate-free and cystine-rich but which contains no sulfhydryl groups, and 3) a short region (≤ 10 kD) which contains all the interchain disulfide bonds(s). Various reports in the literature are consistent with this general picture. Fibronectins, both the cellular form (20, 21, 31) and the plasma form, cold-insoluble globulin (32, 33), are disulfide-bonded dimers. Proteolytic digestion of plasma fibronectin releases a 200-kd fragment (34, 35) and a 27- to 29-kd fragment (35, 36) neither of which contains the interchain disulfides. These results are very similar to some of those reported here for cellular fibronectin.

We are not able to decide conclusively the order of these three regions but presently favor that shown in Fig. 10A (NH₂-200 kD-25 kD-interchain link-CO₂H) for the following reasons: 1) Iwanaga et al. (36) report that the 200-kd fragment of bovine plasma fibronectin has an NH₂-terminal pyroglutamate residue, as does intact fibronectin; 2) preliminary carboxypeptidase Y digestion results suggest that much of the cysteine and the interchain disulfides are removed from the COOH-terminal end releasing large fragments which are not attached by interchain disulfides (23). However, Furie and Rifkin (37) have recently reported that in human plasma fibronectin a 28-kd fragment and the intact molecule both have NH₂-terminal pyroglutamate. This result would favor the model shown in Fig. 10B. Further work will be required to resolve this discrepancy.

Our results bear more directly on the question of distribution of constituents between the different domains. We have presented evidence that at least 66 to 80% of the carbohydrate is located on the 200-kd fragment, but some of our results suggest that all of the carbohydrate may be in this part of the molecule. There is none detectable on the 25-kd fragment. All the carbohydrate appears to be of the complex asparagine-linked type since none is sensitive to endoglycosidase H (38) or to mild alkaline digestion (39). Carter et al. (38) report a single class of glycopeptide of 2,000 daltons after pronase digestion which, on the basis of 5 to 7% carbohydrate, suggests five to seven side chains. In any event, the carbohydrate is concentrated, perhaps exclusively, in the 200-kd region and may be responsible for its relatively resistance to proteolysis. Consistent with this supposition, Olden et al. (39) report that fibronectin which is synthesized in the presence of tunicamycin and lacks carbohydrates is more readily degraded in culture.

The asymmetry of the glucosamine/amino acid ratio across the fibronectin band (Fig. 3) indicates that some chains contain more carbohydrate than others. This has also been suggested for amniotic fluid fibronectin (40). There are reports of double bands for fibronectin, particularly for the plasma form (32, 34, 36, 41, 42) but also for the cellular form (41, 43). These bands could differ in their carbohydrate content. This brings us to a comparison of the present results on cellular fibronectin with those on plasma fibronectin. We have reported elsewhere that fibronectins from these two sources show differences in specific activity for attachment of transformed cells (23) and recently Yamada and Kennedy (42) have also reported differences in biological activity. In comparisons of hamster cellular

³ D. D. Wagner and S. Turco, unpublished data.
and plasma fibronectin, we consistently observe that the plasma form generates two well separated bands after reduction; whereas the cellular form runs as a single band or a very closely spaced doublet. Our results (Fig. 3) suggest that the two members of this doublet from cellular fibronectin may differ in carbohydrate content. The same might be true for the two bands of the plasma form. During tryptic digestion, the difference between cellular and plasma forms persists in the 200-kd fragments (42). Whether this difference in structure of cellular and plasma fibronectins represents a difference in primary sequence or in post-translational modifications, such as glycosylation, remains unclear. It is clear that the overall structures are rather similar.

Whereas the carbohydrate appears concentrated in the 200-kd fragment, the cystine residues of cellular fibronectin are enriched in the other domains of the molecule. The 200-kd fragment which comprises 87% of the molecule contains only 34 to 50% of the half-cystine. This leaves 50 to 66% of the half-cystines (20 to 32 residues) concentrated in 30,000 daltons of each chain. Thus, while fibronectin itself is not particularly enriched in half-cystine (2.3 mole %) this small region is highly enriched (around 10 mole %). This concentration of disulfides in a restricted region is reminiscent of the disulfide knot region of fibrinogen (44). The interchain disulfides appear to be even more localized, being within 10,000 daltons of the end of the molecule. The 25-kd piece probably contains most of the interchain disulfides. Its behavior on gels (Fig. 4) suggests that it contains significant amounts of intrachain disulfide bonding, and it labels relatively heavily with cystine.

There appears to be a single sulfhydryl group per chain, since all the fibronectin and 200-kd fragment are retained on an activated thiol-Sepharose column and estimates using Ellman’s reagent lead to values of one sulfhydryl per chain. Mosesson et al. (32) failed to detect a sulfhydryl group in human plasma fibronectin using NBzS. We have been able to detect one both by NBzS and by the thiol-Sepharose column. The single sulfhydryl of cellular fibronectin is located on the 200-kd fragment (Fig. 6).

The presence of sulfhydryl groups on fibronectin is of considerable interest. Cell surface fibronectin is present both as dimers and in high molecular weight aggregates. Both disaccharide to release monomeric fibronectin on reduction and several other iodinatable polypeptides also dissociate from these complexes (20). On the basis of these earlier results it is not possible to decide whether these complexes represent a non-covalent association of disulfide-bonded fibronectin with other molecules or intermolecular disulfide bonding. The presence of free sulfhydryl groups on dimeric fibronectin makes it possible, even likely, that fibronectin forms intermolecular disulfide bonds, either with itself or with other cell surface proteins. Consistent with this idea is the observation that blocking of the sulfhydryl groups with iodoacetate interferes with binding of fibronectin to cells, although this result could reflect either steric hindrance or interference with intermolecular disulfide bonding. Hence it appears from these results and earlier ones (19) that disulfide bonding of fibronectin is important for attachment and function of this protein at the cell surface.

The structural analysis reported here has identified a domain structure for fibronectin with different regions being enriched for carbohydrate and disulfide bonds and has allowed us to locate the gelatin binding site and the free sulfhydryl group in the 200-kd fragment. Further analyses along these lines should allow location of the several different biologically important binding sites on fibronectin. These include binding sites for cells, collagen, fibrin (33, 36, 45–47), and sulfated glycosaminoglycans or proteoglycans (48, 49).

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