Proc. Natl. Acad. Sci. USA
Vol. 74, No. 7, pp. 2855–2859, July 1977
Cell Biology

Extensive disulfide bonding at the mammalian cell surface

(LETS glycoprotein/fibroblasts/transformation/polyacrylamide gels/intermolecular complexes)

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Communicated by David Baltimore, March 17, 1977

ABSTRACT **Cell surface proteins of cultured cells are disulfide bonded to a greater degree than are total cellular proteins. In particular, the "large external transformation-sensitive" (LETS) protein, a major surface protein, is present almost exclusively in disulfide-bonded complexes including homodimers and also higher aggregates held together by disulfide bonds or noncovalent interactions. Other cell surface proteins also appear to be involved in disulfide bonding, both intramolecular and intermolecular. In virally transformed cells, LETS protein and its disulfide complexes are absent and certain other disulfide-bonded proteins are also not observed.**

Much recent research suggests that the surface of mammalian cells undergoes a change in organization on oncogenic transformation. Electron microscopic studies show altered surface morphology (1, 2). Studies with lectins indicate that the surface proteins of many transformed cells have greater freedom of lateral mobility than do those of their normal counterparts (3). It has been suggested frequently that these changes in surface properties are related to changes in cytoskeleton (4–8). Alterations in the organization of surface proteins also could be due to interactions between surface molecules (9). Whichever is the case, it is necessary to learn more about the molecular nature of the cell surface and the changes in its structure that occur on transformation. Numerous investigations have been made of cell surface proteins in normal and transformed cells (reviewed in ref. 10). The most prominent alteration in cell surface proteins is loss of a large external transformation-sensitive (LETS) glycoprotein (10–14). Few investigations have dealt with interactions between cell surface proteins except in erythrocytes (15–18). We report here that the surface of normal mammalian cells has extensive disulfide bonding, particularly involving the LETS protein.

MATERIALS AND METHODS

Cells and Culture Conditions. The normal hamster cell line, NIL8, and a derivative transformed by hamster sarcoma virus (NIL8-HSV) were cultured as described (11). Cultures were used as confluent monolayers. For labeling, cells were allowed to incorporate [^{35}S]methionine (22 Ci/mmol) or [^{3}H]glucosamine (10 Ci/mol; New England Nuclear) at 50 and 25 μCi/ml. The medium was modified by decreasing the methionine or glucose concentrations to 10% of the usual levels to improve incorporation.

Lactoperoxidase-Catalyzed Iodination. This was carried out on monolayers as previously described (11); this procedure labels only surface proteins in these cells.

Oxidation of Sulfhydryl Groups. Oxidation to disulfide bonds was carried out with *o*-phenanthroline and copper sulfate (17, 18) for 30 min at 22° or with 40 mM hydrogen peroxide for 30 min at 22°.

Abbreviations: LETS, large external transformation-sensitive; NIL8-HSV, normal hamster cell line NIL8 transformed by hamster sarcoma virus.

Preparation of Samples for Electrophoresis. Cells were washed with phosphate-buffered isotonic saline (NaCl or NaI) and lysed in buffer containing 15% (vol/vol) glycerol, 2% (vol/vol) sodium dodecyl sulfate, 50–100 mM Tris·HCl (pH 6.8), 2 mM phenylmethylsulfonylfluoride and 2 mM EDTA to inhibit proteolysis, and 1 mM *N*-ethylmaleimide and 1 mM iodoacetic acid to block free sulfhydryl groups. Cell lysates were boiled for 2 min to denature nucleic acids and proteins. To reduce disulfide bonds, dithiothreitol was added to a concentration of 0.1 M before the boiling step.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis. All gels were 5% (vol/vol) polyacrylamide with a 3% stacking gel (19). For analysis in two dimensions (18, 20), samples were run in 3-mm-diameter cylindrical gels. The first-dimension cells were placed at the top of a slab gel and sealed in place with 0.5% (vol/vol) agarose in electrophoresis buffer. For reduction prior to the second dimension, the agarose contained 10% 2-mercaptoethanol. Markers for the second dimension were applied polymerized in a short cylinder of gel.

After electrophoresis, slab gels were fixed and stained in 7% (vol/vol) acetic acid/50% (vol/vol) methanol/0.0125% (wt/vol) Coomassie blue, destained, and dried down onto filter paper. Radioactivity was detected by autoradiography on x-ray film for ^{125}I or by impregnation with 2,5-diphenyloxazole and fluorography on Kodak blue-sensitive film for ^{3}H and ^{35}S as described (21). Films were scanned on a Zeineh soft-laser densitometer.

RESULTS

Extraction of Surface Proteins Is Promoted by Reducing Agents. In the course of extraction experiments (22), we observed that inclusion of reducing agents in the buffers used to extract intact, lactoperoxidase-iodinated cells led to a marked increase in the efficiency of extraction of surface proteins and, in particular, of LETS protein (Table 1). A similar enhancement, by reducing agents, of extraction of total cellular protein was not observed, indicating that the effect was not due merely to generalized increases in solubility of proteins or to cell lysis. These results suggested that cell surface proteins might be involved in disulfide bonding.

LETS Protein Is Disulfide Bonded at the Cell Surface. Fig. 1 shows the surface proteins of NIL8 cells labelled by lactoperoxidase-catalyzed iodination and analyzed on gels either without or with prior reduction with dithiothreitol. In the reduced samples, the major iodinated band, LETS protein, migrated with an apparent molecular weight of 230,000. If reduction was omitted, the LETS protein band was largely missing and large amounts of iodine were found in a higher molecular weight band, on top of the gel, and at the interface between stacking and running gels. In the reduced samples, little iodine label was trapped on top of the gel (<5%) and less was found at the interface.

Table 1. Extraction of surface and total cellular proteins

Sample	Extractant	% cpm extracted* No DTT	% cpm extracted* +25 mM DTT
From ^{125}I-labeled cell surface proteins	Guanidine·HCl, 5 M	81	95
	Urea, 8 M	43	83
	NaI, 1 M	42	77
	NaI, 2 M	66	82
From ^{125}I-labeled LETS protein	NaI, 1 M	20	78
	NaI, 2 M	42	87
Total cellular proteins	Urea, 8 M	61	56
	NaI, 2 M	64	64

Labeled cells were rinsed and extracted for 30 min at room temperature with 50 mM Tris, pH 6.8/2 mM phenylmethylsulfonylfluoride/2 mM EDTA plus additions as above. Percentage of total incorporated radioactivity extracted was determined by precipitation with trichloroacetic acid. Radioactivity in LETS protein was quantitated by cutting the band from gels.
* Without and with dithiothreitol (DTT).

These results indicate that the LETS protein on iodinated cells is aggregated in disulfide-bonded complexes. The higher molecular weight iodinated band in Fig. 1 migrated as expected of a dimer of LETS protein. Assuming linearity of the plot of logarithm of molecular weight versus migration (23), its molecular weight was 1.97–2.39 times (av., 2.22; $n = 6$) that of the 230,000 molecular weights LETS protein. The rest of the LETS protein in nonreduced samples was found as very high molecular weight aggregates that did not enter the gels. Electrophoresis on low percentage gels (2–4%) showed that trimers, tetramers, and complexes up to octomers were not found (data not shown). Almost all the aggregates of LETS protein moved to the 230,000 dalton band on reduction, indicating that the only covalent crosslinks involved in forming the aggregates were disulfide bonds.

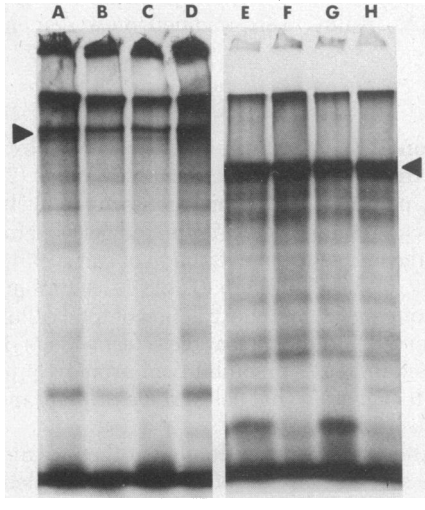

FIG. 1. Polyacrylamide gel electrophoresis of iodinated NIL8 cells with and without sulfhydryl blocking and with and without reduction. Autoradiogram of slab gel. Dishes of NIL8 cells were rinsed, preincubated for 30 min in phosphate-buffered saline with additions as below, rinsed, and iodinated. Iodinated cells were analyzed either before (lanes A–D) or after (lanes E–H) reduction with 0.1 M dithiothreitol. Lanes A and E, control (no additions during incubation). Lanes B and F, 1 mM *p*-chloromercuribenzenesulfonate. Lanes C and G, 1 mM iodoacetic acid. Lanes D and H, 1 mM *N*-ethylmalemide. Arrows mark position of LETS monomer (230,000 daltons) and dimer (460,000 daltons).

Table 2. Distribution of ^{125}I radioactivity between aggregates and dimers and monomers of LETS protein

Treatment of cells*	Percentage distribution of cpm† Top of gel	Gel interface	Dimer	Monomer
Before iodination				
None (control)	38	28	27	7
IAA, 1 mM	32	38	25	5
NEM, 1 mM	31	34	28	8
IAA + NEM, 1 mM each	30	30	25	6
o-Phenanthroline plus copper‡	39	38	17	5
After iodination				
DTT, 0.1 M	3	17	9	71
3% mercaptoethanol followed by *o*-phenanthroline plus copper‡	72	14	5	9

* Confluent dishes of NIL8 cells were iodinated with or without preincubation or postincubation at 22° for 30 min with additions as shown. IAA, iodoacetic acid; NEM, *N*-ethylmaleimide; DTT, dithiothreitol.
† Cells were harvested in a lysis buffer containing 1 mM NEM and 1 mM IAA and run on gels without reduction (compare Fig. 2). The radioactivity in each of the four regions was determined by cutting and counting and is expressed as a percentage of the sum of the four regions. The major labeled component in the aggregates was LETS protein but other iodinated proteins contributed counts so the values are not strict representations of the distribution of LETS protein. They do indicate the degree of general disulfide cross linking.
‡ *o*-Phenanthroline–copper complex promotes sulfhydryl group oxidation to disulfides. See *Materials and Methods*.

Because lactoperoxidase-catalyzed iodination involves the production of hydrogen peroxide, the possibility arose that the disulfide bonds observed were artifactual. That this was not the case was shown in several ways. NIL8 cells were treated with sulfhydryl blocking reagents before and after iodination. Pretreatment for 30–60 min with 0.1–10 mM *N*-ethylmaleimide, iodoacetic acid, or *p*-chloromercuribenzenesulfonic acid or a mixture of the first two at 1 mM each did not affect the results. In each case, essentially all the LETS protein was found as dimers or higher aggregates. No significant differences in distribution of radioactivity were found (Fig. 1 and Table 2). Other details of the pattern also remained unchanged by these pretreatments (see below). Because these treatments lead to extensive blocking of sulfhydryl groups (refs. 24 and 25; unpublished data), formation of disulfide bonds during iodination is not responsible for the patterns observed. In an additional control experiment, cells were iodinated, treated with 3% 2-mercaptoethanol to reduce disulfide bonds, washed, and then oxidized with either hydrogen peroxide or *o*-phenanthroline–copper. The pattern of ^{125}I-labeled bands observed after this treatment was different from the pattern observed in control cells, indicating that the control pattern was not the result of random oxidation. The control pattern appeared to be fairly stable in that it was not greatly affected by oxidation if the reduction step was omitted.

Finally, the existence of dimers of LETS protein was shown also by metabolic labeling with methionine or glucosamine. Fig. 2 shows analysis of conditioned medium from two cell types.

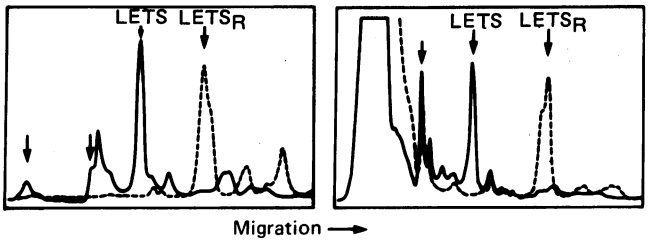

FIG. 2. Analysis of conditioned medium after labeling for 24 hr. (*Left*) Secondary chick embryo fibroblasts with [^{35}S]methionine. (*Right*) NIL8 hamster cells with [^{3}H]glucosamine. Samples were analyzed on gels either without (—) or with (- - -) reduction. After fluorography, the x-ray films were scanned with a densitometer. LETS$_R$, 230,000 dalton monomer of LETS.

No 230,000 dalton monomer was observed in the absence of reduction. Analogous results were observed with whole-cell lysates. In some gels, after reduction the LETS protein band appeared as a doublet, suggesting the possibility of differences between the two halves of the dimer (Fig. 2).

Other Proteins Participate in Disulfide-Bonded Complexes. The extraction results showed that surface proteins other than LETS protein were also more readily extracted in the presence of reducing agents (22). To examine further the nature of the disulfide-bonded complexes among surface proteins, iodinated cells were analyzed on two-dimensional gels, nonreduced in the first dimension and reduced in the second (18, 20). Proteins unaffected by reduction migrated onto a diagonal, whereas those with disulfide bonds migrated off the diagonal and their position provided information on the nature of the complexes (Fig. 3*A*). In addition to the strong band of radioactivity along the diagonal, numerous spots were observed off the diagonal, both above and below it. Control gels run either nonreduced in both dimensions (Fig. 3*C*) or reduced in both dimensions (Fig. 3*D*) showed no off-diagonal spots, proving that the off-diagonal spots in Fig. 3*A* were caused by the reduction step between the two electrophoresis stages.

The spots above the diagonal presumably represent proteins with intramolecular disulfide bonds that only after reduction are able to adopt the rod-shaped form characteristic of sodium dodecyl sulfate–protein complexes (26). This leads to decreased mobility in the second dimension. Bovine serum albumin,

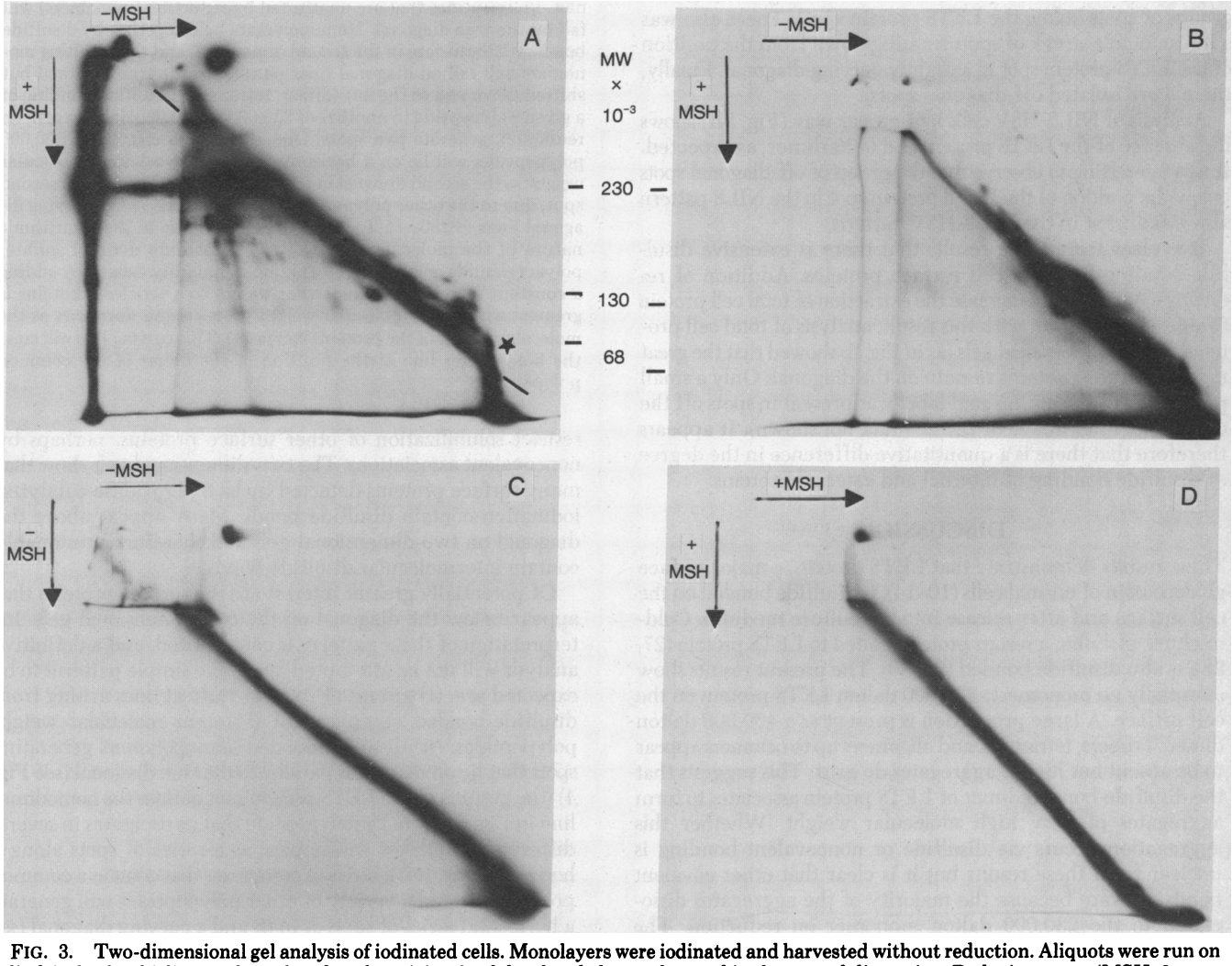

FIG. 3. Two-dimensional gel analysis of iodinated cells. Monolayers were iodinated and harvested without reduction. Aliquots were run on cylindrical gels which were then placed at the origin of a slab gel and electrophoresed in the second dimension. Reducing agent (MSH, 2-mercaptoethanol) was present as shown. (*A*) NIL8 cells; first dimension nonreduced, second dimension reduced. Note spots off diagonal. Star marks position of bovine serum albumin. Line shows true diagonal. (*B*) NIL8-HSV cells; first dimension nonreduced, second dimension reduced. Note the absence of the group of off-diagonal spots at upper left. Molecular weight markers in second dimension are LETS protein (230,000), β-galactosidase (130,000) and bovine serum albumin (68,000). (*C*) NIL8 cells; both dimensions without reduction. (*D*) NIL8 cells; both dimensions with reduction. Note absence of off-diagonal spots in (*C*) and (*D*).

which has numerous internal disulfide bonds, shows this property and is often seen in our gels because it is a contaminant from the culture medium.

The second group of off-diagonal spots was below the diagonal, where one expects to find proteins that, prior to reduction, were in disulfide-bonded complexes. Fig. 3A shows many protein spots in this region. Particularly prominent is LETS protein, little of which is on the diagonal. There is a large spot in the position expected for the LETS dimer (coordinates 460,230) and a streak along the 230,000 line in the second dimension with large accumulations of radioactivity at positions corresponding to the top and interface of the first gel.

Of particular interest is the presence of other spots below the diagonal. The positions of these spots were reproducible in a large number of gels. Pretreatment of cells with sulfhydryl blocking reagents prior to iodination did not affect the pattern of off-diagonal spots, indicating that they were not artifactual (data not shown). The spots below the diagonal exhibited a number of different patterns. Some were arranged in vertical lines, as at the positions corresponding to the top and the interface of the first dimension gel. A second type of pattern consists of series of spots both on lines parallel to the diagonal and on diagonal lines not parallel to the main diagonal (e.g., the groups of spots below the LETS protein spot). There also was a reproducible streak of spots running down from the position of the LETS protein spot in a slightly curving diagonal. Finally, there were isolated off-diagonal spots.

Analysis of NIL8-HSV cells in a similar way (Fig. 3B) shows the absence of the LETS protein and of its dimer, as expected. It was interesting to observe that the group of off-diagonal spots below the position of the LETS protein spot in the NIL8 pattern also was absent in the NIL8-HSV pattern.

It is clear from these results that there is extensive disulfide-bonding between cell surface proteins. Addition of reducing agents did not increase the extraction of total cell protein (Table 1). Consistent with this result, analysis of total cell proteins on two-dimensional gels (as in Fig. 3) showed that the great majority of the proteins remain on the diagonal. Only a small proportion of the amino acid label was present in spots off the diagonal, either above or below (data not shown). It appears therefore that there is a quantitative difference in the degree of disulfide bonding of internal and external proteins.

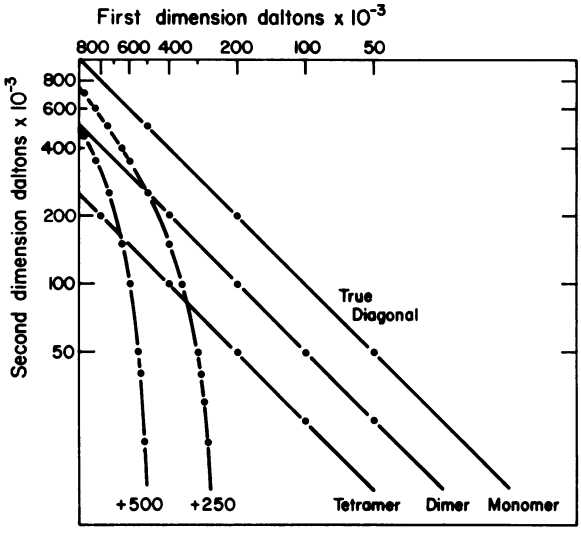

FIG 4. Predicted migration of various complexes in two-dimensional gels. Figure represents a hypothetical two-dimensional gel, nonreduced in first dimension and reduced in the second, with true linearity on a molecular weight-versus-migration semilogarithmic plot. Polypeptides that are unaffected by reduction (monomers) will fall on the true diagonal. Homopolymers held together by disulfide bonds will dissociate in the second dimension, and the resulting monomers will fall on diagonal lines parallel to the true diagonal but shifted down and to the left (dimer, tetramer). Disulfide bonding of a given polypeptide to another of 250,000 or 500,000 daltons will, on reduction, generate two spots. One, due to the 250,000 or 500,000 polypeptide, will lie on a horizontal line at the relevant molecular weight in the second dimension (not shown in the figure). The second spot, due to the other polypeptide, will fall on one of the curving diagonal lines (+250, +500). The curvature is due to the logarithmic nature of the molecular weight scale on sodium dodecyl sulfate/polyacrylamide gels (the perturbation in mobility caused by adding a constant increment of molecular weight to a series of proteins is greatest with lower molecular weight proteins and decreases as the molecular weight of the proteins increases). This curving line will cross the homodimer line at the position of the dimer of the common polypeptide.

DISCUSSION

These results demonstrate that LETS protein, a major surface glycoprotein of normal cells (10–14), is disulfide bonded on the cell surface and after release into the culture medium. Cold-insoluble globulin, a serum protein related to LETS protein (27, 28), is also disulfide bonded (29, 30). The present results show essentially no monomeric 230,000 dalton LETS protein on the cell surface. A large proportion is present as a 460,000 dalton dimer. Trimers, tetramers, and oligomers up to octomers appear to be absent but higher aggregates do exist. This suggests that the disulfide-bonded dimer of LETS protein associates to form aggregates of very high molecular weight. Whether this aggregation occurs via disulfide or noncovalent bonding is unclear from these results but it is clear that other covalent bonds are rare because the majority of the aggregates dissociated to the 230,000 dalton monomer on reduction. The aggregation of LETS protein is consistent with the observation by immunofluorescent microscopy that it is located in a fibrillar network (22, 28, 31–33).

The increased extraction of other surface proteins by reducing agents (22) suggests that they also participate in disulfide bonding or that the disulfide-bonded LETS protein aggregates restrict solubilization of other surface proteins, perhaps by noncovalent associations. The two-dimensional gels show that many surface proteins detected by lactoperoxidase-catalyzed iodination contain disulfide bonds. Many appear above the diagonal on two-dimensional gels and therefore presumably contain intermolecular disulfide bonds.

Of potentially greater interest are the surface proteins that appear below the diagonal on the two-dimensional gels. Interpretation of these patterns is complicated, and a definitive analysis will not be attempted here. The simple patterns to be expected are: (*i*) groups of spots in vertical lines arising from disulfide-bonded complexes of different molecular weight polypeptides; (*ii*) disulfide-bonded homopolymers generating spots that lie on diagonals parallel to the true diagonal (see Fig. 4) (the position of the LETS protein spot defines the homodimer line in Fig. 3A); (*iii*) a polypeptide that participates in several different complexes will appear as a series of spots along a horizontal line; (*iv*) a series of complexes that contain a common polypeptide and a variety of other polypeptides will generate a horizontal series of spots as in *iii* and a curving diagonal (see Fig. 4); and (*v*) a complex between an iodinated polypeptide and an unlabeled one will generate an off-diagonal spot without a partner.

Spots that appear to conform to several of these patterns are observed in Fig. 3A, suggesting that many surface proteins participate in various disulfide-bonded complexes. In theory,

the information contained in the coordinates of the spots and the patterns in which they are arranged should allow one to determine the nature of the complexes. However, this analysis is complicated by several factors, including failure of the linear relationship between molecular weight and mobility because of the glycoprotein nature and the very high molecular weight of many of the polypeptides and because of possible anomalous migration due to intramolecular and intermolecular disulfide bonds. The possible occurrence of proteolytic "nicking" within disulfide-bonded polypeptides which will only be revealed in the second dimension raises another complication.

Because of these factors one cannot, as yet, deduce the nature of all the complexes revealed by two-dimensional gels such as in Fig. 3A. Some conclusions are clear, such as the nature of the LETS dimer and that other surface proteins are involved in disulfide bonding. Other features of the pattern are subject to several interpretations. For example, the curving diagonal descending from the LETS dimer spot could arise from a series of heteropolymers (pattern *iv*) or from "nicking" within a dimer of LETS protein. Solutions to these complications do exist. The pattern can be simplified, as discussed elsewhere (22), by selective extraction procedures or by selecting immunologically those complexes involving particular proteins, and it seems probable that further analysis will provide more information concerning the intermolecular complexes. The results already reveal the existence of various complexes and suggest that disulfide bonding is more prevalent at the cell surface than internally.

Scattered earlier reports have indicated the existence of disulfide bonds in several membrane proteins: transplantation antigens (34, 35), surface immunoglobulins (36, 37), and oncornavirus glycoproteins (38). Ando and Steiner (25) reported evidence for intramolecular disulfide bonds in platelet membranes but found no intermolecular bonds. The present data suggest that disulfide bonding may contribute extensively to interpolypeptide interactions at the cell surface.

It is of interest that both the LETS dimer and a complex of other off-diagonal surface polypeptides are missing in transformed cells (Fig. 3B). One interpretation is that these spots are related to the presence of LETS protein in some way, possibly by disulfide bonding. However, proof of this hypothesis is lacking. As mentioned earlier, the increased extraction of several surface proteins from normal cells in the presence of reducing agents suggests that they may be disulfide bonded or associated noncovalently with some other disulfide-bonded complex. The possibility of intermolecular complexes in the cell surface is suggested by various results (8–10). Furthermore, there are implications that intermolecular organization may be altered upon oncogenic transformation. The results reported here are consistent with these suppositions.

Note Added in Proof. After submission of this manuscript we learned that Keski-Oja *et al.* (39) have also observed the dimeric nature of LETS protein and Phillips and Agin (40) have reported disulfide-bonded heterocomplexes in platelet membranes.

We thank Frank Solomon for his helpful criticism. This work was supported by Grants R01 CA 17007 and P01 CA 14051 from the National Cancer Institute, U.S. Public Health Service.

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