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STUDIES OF A LARGE TRANSFORMATION-INCREASED MEMBRANE PROTEIN IN THE BHK21 CELL SYSTEM

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Summary

We have recently described in BHK cells a plasma membrane protein of molecular weight 177 000, which is significantly increased in Hamster Sarcoma Virus-transformed cells (Lage-Davila, A. and Montagnier, L. (1977) Biochem. Biophys. Res. Commun. 79, 577—584). We present now a study of proteins from purified plasma membrane fractions in the same pair of clones. Solubilization conditions, cross-linking experiments, metabolic labelling and enzymatic radioiodination allow to characterize this 177 000 transformation-increased protein as an integral membrane glycoprotein partially exposed at the outer cell surface. Additional information on other membrane proteins in this system is also given.

Introduction

Previous work from this laboratory has called attention to changes in integral membrane proteins which accompanied viral transformation in several in vitro systems. These changes are expressed at the ultrastructural level by a significant increase in density of intramembranous particles in freeze-fractured plasma membranes [1]. We have also recently reported [2] an increase in a large membrane protein (M_r = 177 000) in BHK21/13 cells transformed by the B34 strain of Hamster Sarcoma Virus (HaSV), which can be distinguished from myosin and collagen precursors.

From growth properties in agarose medium, BHK21 cells can be considered as having undergone a first stage of transformation [3]. A second stage of transformation is achieved by action of oncogenic viruses, including HaSV. The

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Abbreviations: M_r, molecular weight. It should be always understood as apparent molecular weight based in electrophoretic mobility in dodecyl sulphate-polyacrylamide gels.

present paper brings further data on plasma membrane proteins of BHK21 cells and their HaSV-transformed derivative. Solubilization conditions, cross-linking experiments, metabolic labelling, and external enzymatic radioiodination allow a closer picture of the 177 000 transformation-increased protein which can be proposed as a provisional characterization until purification procedures will provide a more complete view. All results are consistent with the previously proposed view of an increase, in the most transformed clone, of a large, integral, and partially exposed membrane glycoprotein.

From the various experimental approaches used, additional information was also gained on other membrane proteins of BHK21 cells.

Materials and Methods

Materials

All chemical reagents were of analytical purity. L-[^{35}S]Methionine and radioactive iodine ($Na^{125}I$) were purchased from Amersham Radiochemical Center. D-[^{14}C]Glucosamine was obtained from the C.E.N., Saclay, France. Enzymes and phenylmethylsulfonylfluoridine for iodination experiments were purchased from Calbiochem, Diisopropylfluorophosphate was obtained from B.D.H. and Acrylamide and *N,N'*-methylenebisacrylamide from Serva.

Cells, plasma membrane purification and electrophoresis

An asparagine-dependent subclone, C13/8, of BHK21/13 cells was used as untransformed control [4]. The transformed clone was the HS5 clone [2] isolated in agarose medium containing dextran sulphate [5], after infection of C13/8 cells with B34 strain of Hamster Sarcoma Virus [6]. This clone is not virus producing. Plasma membrane purification and electrophoresis in dodecyl sulphate-containing polyacrylamide gels were performed as previously described [2]. Slab gels and autoradiograms were scanned in a VERNON photometer-integrator scanner.

Solubilization studies

For assays of solubilization, plasma membrane fractions were pelleted at 51 000 × *g* for 1 h. Membrane pellets were carefully resuspended by means of a Dounce homogenizer (tight pestle) in the solubilizing solution at a concentration about 1 mg of protein/ml and incubated at 37° C for 1 h. Solubilization was attempted with the following solutions:
(A) 1 mM EDTA, 5 mM glycine, pH 9.5 [7]; (B) 0.45 M KH_2PO_4/KCl, pH 6.5 [8]; (C) 3 mM EDTA, 20 mM Tris-HCl, pH 8.0; (D) 0.5% Triton X-100, 5 mM sodium phosphate buffer, pH 7.0; (E) 1.0% Triton X-100, 20 mM Tris-HCl, pH 8.0; (F) 1.0% Triton X-100, 5% β-mercaptoethanol, 20 mM Tris-HCl, pH 8.0, and (G) 1.0% Triton X-100, 3 mM EDTA, 20 mM Tris-HCl, pH 8.0. All solutions also contained 20 μg/ml of diisopropylfluorophosphate. Following incubation, undissolved material was discarded by centrifugation at 200 000 × *g* for 50 min. The supernatant was called the 'solubilized fraction'.

For electrophoretic analysis, solubilized proteins were then precipitated from the supernatant with cold acetone. Two volumes of cold acetone were added and the mixture was incubated for 20 min at −20° C and then

centrifuged at 10 000 rev./min for 1 h at −20° C in an RCB2 Sorvall centrifuge. The supernatant, containing most of the detergent was discarded and the pellet resuspended in electrophoresis sample buffer (3% sodium dodecyl sulphate/5% mercaptoethanol/20% glycerol/0.15 M Tris-HCl, pH 6.8). Acetone precipitation could not be used with solubilizing solution B because of concomitant precipitation of salts. In these experiments KCl-extracted proteins were precipitated by making the solution 30% in cold trichloroacetic acid. Precipitated proteins were centrifuged at 10 000 rev./min for 1 h at 0° C and the pellet washed twice with cold ethylic ether to eliminate trichloroacetic acid residues, before final resuspension in electrophoresis sample buffer.

Oxidative cross-linking reaction

Two membrane pellets containing 200 μg protein each were resuspended by strong agitation in 20 mM Tris-HCl (pH 7.4) containing 100 μM *o*-phenanthroline and 20 μM $CuSO_4$ [9] and incubated at room temperature with continuous agitation for 1 and 5 min, respectively. Reactions were stopped by direct addition of two volumes of a solution containing 6% sodium dodecyl sulphate, 15% glycerol, 0.015% bromophenol blue (tracking dye) and 0.17 M Tris-HCl (pH 6.8) and heating at 100° C for 3 min. A control membrane pellet was similarly treated but without *o*-phenanthroline and $CuSO_4$ in the buffer. A reversibility control was also included which consisted as a sample cross-linked for the maximal time and dissolved for electrophoresis in the same stopping buffer but containing also 5% β-mercaptoethanol.

Metabolic labelling

Cells were grown in minimal essential medium prepared with 1/5 of normal methionine concentration (0.04 M instead of 0.2 mM), without Bactotryptose and supplemented with L-serine (10 μg/ml) and L-asparagine (15 μg/ml). Radioactive methionine (1050 Ci/mM) was added at a final concentration of 10 μCi/ml. After 48 h of culture, cells were harvested by trypsinization and used for plasma membrane purification. In these conditions, intensity of label reflects for each protein its steady-state amount. A specific radioactivity of $1.6 \cdot 10^6$ cpm/mg protein could be obtained in cell homogenates. About 4% of the label was recovered in the plasma membrane fraction with the same specific radioactivity which shows that methionine label is neither concentrated in, nor excluded from, plasma membranes. For metabolic labelling with [^{14}C]glucosamine (57 Ci/M) the cells were cultured for 48 h in modified Eagle's minimal essential medium [10] containing 0.4 μCi/ml of the isotope (12 μCi/bottle). These cultures were not trypsinized for cell harvesting. Instead, cells were detached by incubation for 10 min at 37° C in phosphate-buffered saline solution containing 0.54 mM EDTA. After plasma membrane purification and electrophoresis of membrane fractions the gels were first stained for proteins with Coomassie Blue, photographed, and then treated for scintillation autoradiography as described by Bonner and Laskey [11]. The position of molecular weight markers was labelled with ink containing [^{35}S]methionine.

Ultracentrifugation of solubilized membrane proteins

[^{35}S]Methionine-labelled plasma membrane fractions were extracted with 1%

Triton X-100, 5% β-mercaptoethanol, 3 mM EDTA, 5 mM sodium phosphate buffer pH 8.0, at 37°C for 1 h. About 55—60% radioactivity was solubilized and this extract was used for ultracentrifugation experiments in sucrose gradients.

An aliquot of Triton-solubilized membrane protein containing 50 000 cpm of [^{35}S]methionine was layered on a linear 15—25% w/w sucrose gradient. Sucrose solutions were made in 50 mM Tris-HCl (pH 7.4) containing 0.1 M NaCl, 0.05% Triton X-100, 3 mM EDTA and 0.5% β-mercaptoethanol. The gradient was centrifuged at 50 000 rev./min (245 500 × g) for 17 h at 5°C in a SW-56 Beckman rotor. About 25 fractions of 0.2 ml were collected from the bottom. Fractions corresponding to radioactivity peaks were pooled, dialyzed overnight against distilled water at 4°C, lyophilized and resuspended in electrophoresis sample buffer. After electrophoresis polyacrylamide gels containing [^{35}S]methionine-labelled proteins were dried in a BioRad Gel Slab Dryer and exposed to Kodak RP Royal X-Omat autoradiographic film for 8—15 days at room temperature.

Enzymatic radioiodination of externally exposed proteins

Externally exposed proteins were labelled by the lactoperoxidase catalyzed iodination essentially as described by Hynes [12]. Confluent cultures in 70 cm^2 flasks were used after 72 h of culture with an intermediate change of medium. After removal of medium the cells were washed three times with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline and then 2 ml of phosphate-buffered saline containing 5 mM glucose and 400 μCi of carrier-free Na^{125}I were added to each flask followed by 40 μg of lactoperoxidase (0.56 I.U.) and 0.2 units of glucose oxidase to start the reaction. Iodination was allowed to continue for 10 min at room temperature, then the medium was removed and the cells were washed three times with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline containing 2 mM phenylmethylsulfonylfluoridine. Iodinated cells were scraped with a rubber policeman and used immediately for plasma membrane purification or directly resuspended in electrophoresis sample buffer for whole cell protein analysis. After electrophoresis ^{125}I-labelled proteins were detected in slab gels by autoradiography of dried gels on Kodak RP Royal X-Omat films. During the plasma membrane purification, iodine distribution was monitored by liquid scintillation counting.

Results

Solubilization of HS5 plasma membrane proteins

All membrane proteins dissolved upon heating at 100°C for 2—3 min in the presence of 3% sodium dodecyl sulphate and 5% β-mercaptoethanol, allowing electrophoretic resolution. Searching for milder solubilization conditions, we tested the seven solutions described in Materials and Methods. Fig. 1 shows the typical HS5 and C13/8 membrane electrophoretic pattern and the electrophoregrams of proteins solubilized by high KCl concentration (B-solution), EDTA at low ionic strength (A-solution) and 1% Triton X-100 (E-solution). More concentrated 3 mM EDTA (C-solution) gave the same results as 1 mM EDTA. Triton X-100 extracted the same set of proteins either in phosphate (D-

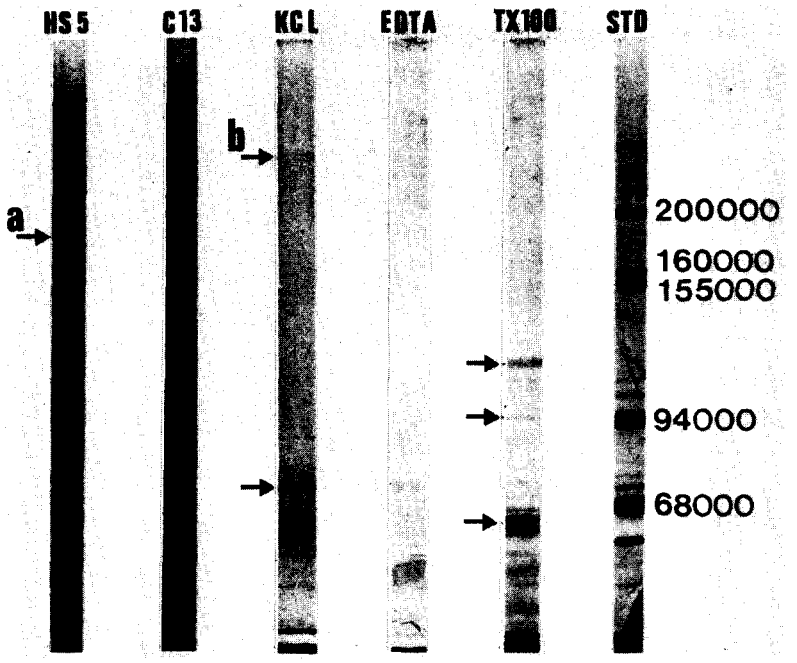
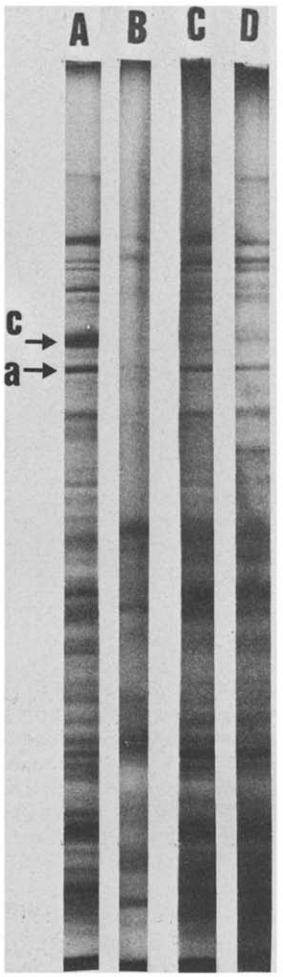


Fig. 1. Solubilization of membrane proteins. From left to right, the first two lanes are typical slab gel electrophoretic patterns of plasma membrane fractions from transformed (HS5) and untransformed (C13) cells. Arrow a indicates the 177 000 transformation-increased protein. The following gels correspond to material extracted respectively with KCl (B-solution), EDTA (A-solution) and Triton X-100 (E-solution). See text for the meaning of arrows. The last gel (STD) shows the position of standard molecular weight markers: chicken muscle myosin (M_r = 200 000); *E. coli* RNA-polymerase (160 000 and 155 000), rabbit muscle phosphorylase *A* (94 000) and bovine serum albumin (68 000). Sense of migration is downward.

solution) or in Tris buffered (E-solution). Two high molecular weight proteins (M_r = 230 000 and 240 000) visible as a double band in HS5 and C13/8 membrane electrophoregrams were extracted only with high KCl (arrow b). Another single band (M_r = 66 000, arrow) was also extracted in these conditions and also with 1 mM EDTA. Triton X-100 extracted a set of membrane proteins of M_r below 116 000: the arrows indicate the most prominent ones. None of the major high molecular weight membrane proteins, with the exception of the two salt-soluble ones, could be extracted by these solubilization procedures and were invariably recovered in the non-solubilized pellet: among them, was the 177 000 transformation-increased protein (arrow a).

Fig. 2 compares the protein set extracted from HS5 membranes with Triton X-100 alone (gel B) and supplemented with 5% β-mercaptoethanol (gel C) or with 3 mM EDTA (gel D). The whole set of high molecular weight proteins, with the exception of a 195 000 band (arrow c), passed into the non-sedimentable supernatant when Triton X-100 solution was supplemented with β-mercaptoethanol. A similar effect could be achieved by adding 3 mM EDTA to the Triton solution. The 177 000 transformation-increased protein readily went into solution with 1% Triton X-100, 5% β-mercaptoethanol. The effect of mercaptoethanol in allowing detergent solubilization of high molecular weight membrane proteins from HS5 cells behaved as 'all or none'. In the absence of



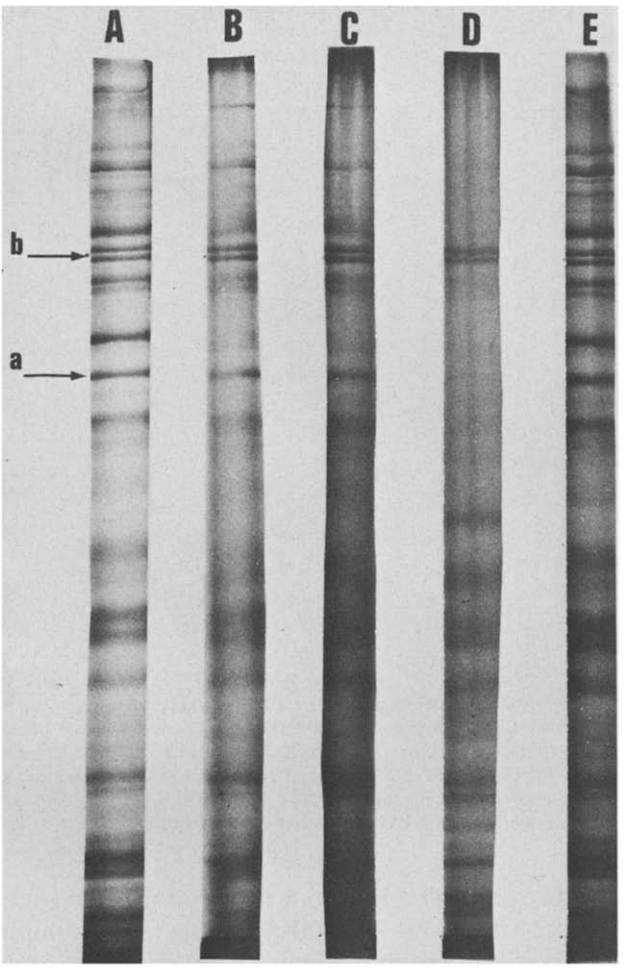


Fig. 2. Solubilization of membrane proteins with Triton X-100 solutions. Slab gel electrophoresis of a purified membrane sample (A) and of material solubilized from purified membranes by means of 1% Triton X-100 alone (B) and supplemented with 5% β-mercaptoethanol (C) or 3 mM EDTA (D). Arrow a indicates the 177 000 transformation-increased protein; arrow c indicates the non-extractable 195 000 protein.

Fig. 3. Oxidative cross-linking experiment. Slab gel electrophoresis of a purified membrane sample under usual reductive conditions (A) and without β-mercaptoethanol in the sample buffer (B). Lanes C and D are electrophoregrams of membrane samples previously treated with *o*-phenanthroline/$CuSO_4$ for 1 and 5 min, respectively. Lane E (reversibility control) corresponds to a sample treated as in D and then dissolved in sample buffer containing β-mercaptoethanol. Arrow a indicates the 177 000 transformation-increased protein. Arrow b indicates the two cross-linking resistant bands.

mercaptoethanol, Triton X-100 solubilized 30% of membrane protein. The Triton/mercaptoethanol solution solubilized 60% of membrane protein (as estimated both from Lowry protein measurements and from [^{35}S]methionine radioactivity counts) and 90% of [^{14}C]fructose label.

Oxidative cross-linking experiments

The requirement of β-mercaptoethanol for detergent solubilization of HS5

membrane proteins and the fact that a group of membrane proteins behaved in a concerted manner in solubilization experiments raised the possibility of the existence of oligomeric associations. We were thus led to investigate the effects of the opposite chemical conditions, that is, the full oxidation of sulfhydryl groups catalyzed by the chelate complex *o*-phenanthroline-$CuSO_4$. It has been shown that this complex catalyzes the air oxidation of SH groups to disulphides [13]. Fig. 3 shows the electrophoregrams of cross-linked membrane samples. High molecular weight membrane proteins rapidly disappeared from the pattern after SH oxidation. It is noteworthy that the two KCl-extractable proteins (arrow b) were completely resistant to cross-linking in these conditions. Sensitive proteins were remarkably similar to those requiring β-mercaptoethanol for detergent solubilization. The extensively cross-linked sample gave an electrophoregram similar to that of the Triton (alone) extract, as can be seen by comparing gel D from Fig. 3 with gel B from Fig. 2. Extensive cross-linking was fully reversible by addition of β-mercaptoethanol after cross-linking reaction had taken place.

Fig. 4 shows the ultraviolet absorbance scannings of electrophoregrams of cross-linked samples after 1 and 5 min reaction, as compared with the control

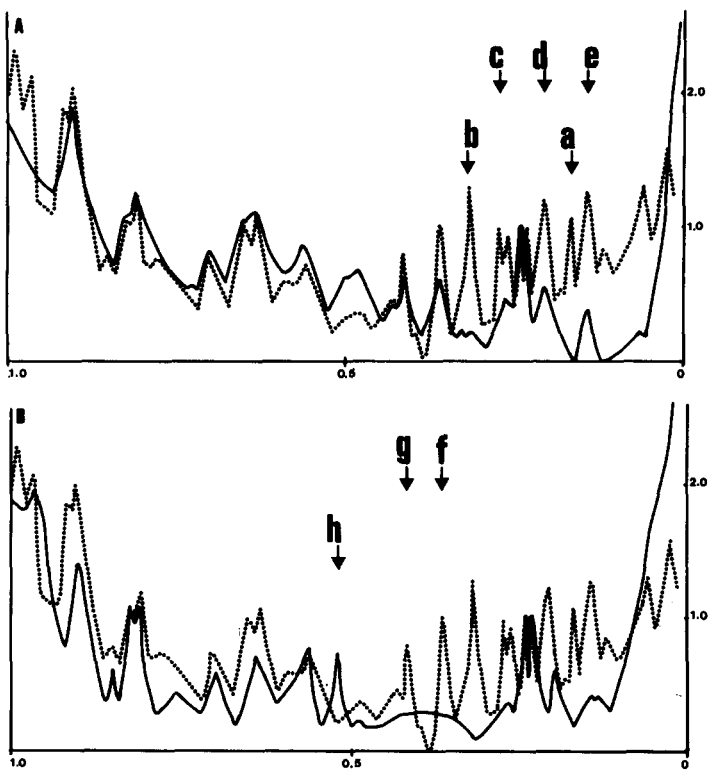
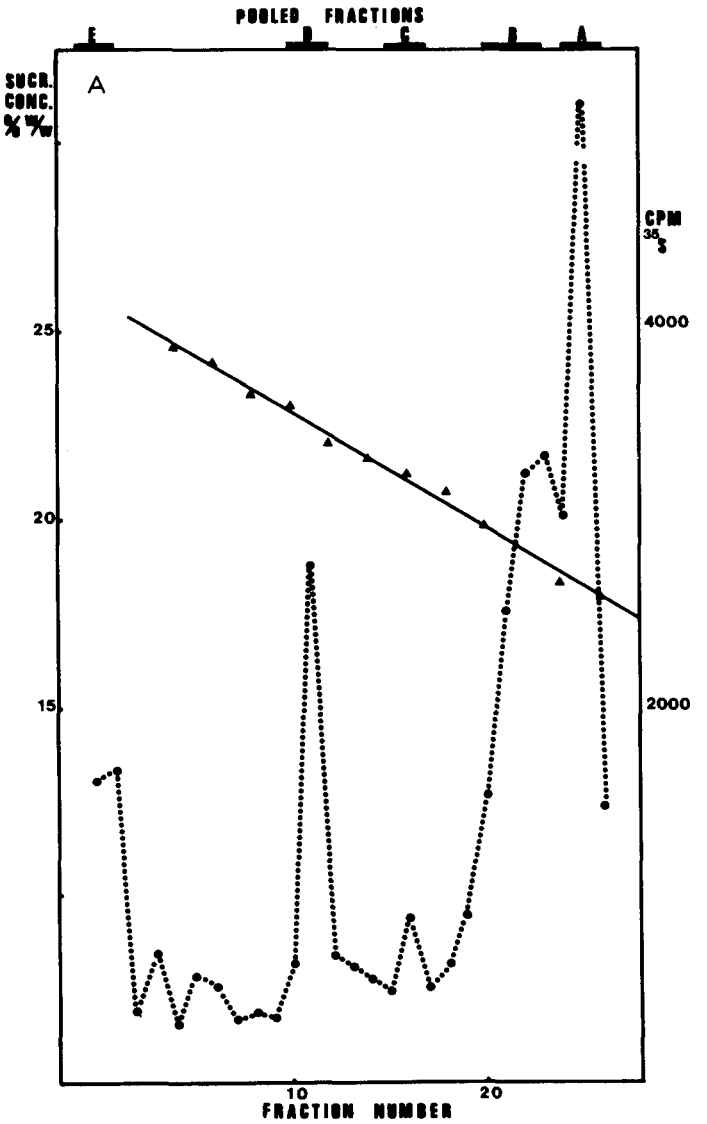


Fig. 4. Oxidative cross-linking experiment. Ultraviolet absorbance scannings of electrophoregrams of control (· · · · · ·) membrane samples as compared with samples exposed to the cross-linking reaction (———) for 1 (4A) and 5 4(B) min. Sense of migration is from right to left. Abscissa are electrophoretic R_F values. Ordinates are relative absorbances, obtained by dividing the observed absorbance by the absorbance of the 235 000 cross-linking resistant band. See text for the meaning of arrows.

sample. Several degrees of sensitivity can be distinguished. Arrows a and b indicate two bands (M_r = 275 000 and 195 000) which completely disappeared in the first minute of reaction. Arrows c, d and e indicate three other bands (M_r = 220 000, 255 000 and 290 000) which greatly decreased. Between the first two, the two cross-link resistant bands can be seen. After the 5 min reaction (Fig. 4B) in addition to the previously erased bands, two other proteins disappeared (arrows f and g). The first one (arrow f) corresponds to the 177 000 transformation-increased protein. An additional band, normally not present in fully reduced membrane electrophoregrams (arrow h) appeared after 5 min of cross-linking in a position corresponding to a nominal molecular weight of 125 000. Its identity was not further investigated.



Sedimentation behaviour of solubilized membrane proteins

Fig. 5A shows the sedimentation pattern of Triton/mercaptoethanol-extracted proteins through a 15—25% w/w linear sucrose gradient. Proteins had been metabolically labelled with [^{35}S]methionine prior to plasma membrane purification. The protein composition of the five major radioactivity peaks can be seen in autoradiograms shown in Fig. 5B. Peak A which corresponds to the initial sample layer showed no band in the autoradiogram, indicating that its radioactivity came from small methionine-containing molecules. Peak B was a large zone spreading through five fractions which contained a group of proteins of molecular weight no higher than 100 000. The small peak C contained mainly two polypeptides of M_r = 116 000 and 100 000. The greatest set of membrane proteins was found in the sharp peak D. Proteins in peak D were of M_r varying from 50 000 to 300 000. The absence of physical separation of

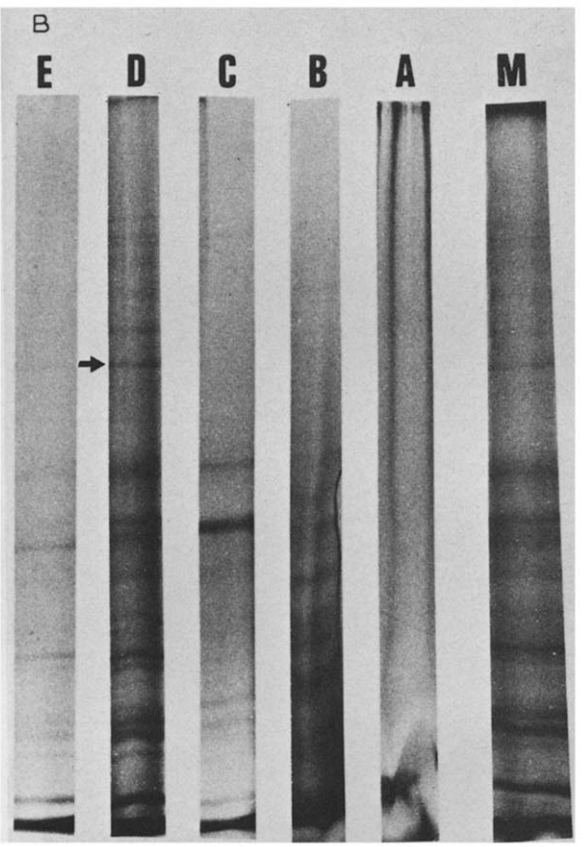


Fig. 5. (A). Density gradient resolution of solubilized membrane proteins. Sense of sedimentation is from right to left. The slope of the sucrose gradient (left ordinate, △———△) and the distribution of [^{35}S]-methionine radioactivity (right ordinate, ○·····○) are indicated. Bars A—E on the top abscissa indicate the position of the fractions pooled for electrophoretic analysis. (B). Protein composition of pooled fractions. Lanes A—E show the electrophoregrams [^{35}S]methionine autoradiograms of respective fractions pooled from the gradient. Lane M shows the electrophoretic pattern of the initial sample of solubilized membrane proteins. The arrow indicates the position of the 177 000 protein.

these proteins in the gradient on the basis of their molecular sizes suggests that they are all included in a common heterogeneous structure, presumably a detergent micelle. Peak E is the bottom of the tube, including presumably aggregates.

Glycosylated membrane proteins

Glucosamine label concentrated two-fold in plasma membranes from C13/8 cells and three times in plasma membranes from HS5 cells. Fig. 6 shows the autoradiograms of plasma membrane glycoproteins metabolically labelled with [^{14}C]glucosamine. Nine major glycosylated bands could be detected in plasma membranes from HS5 cells. Several differences between untransformed C13/8 and transformed HS5 cells could be seen in electrophoretic patterns of glycosylated plasma membrane proteins. We focussed our attention on three of them. First, there is a glucosamine label at the position of the 177 000 transformation-increased protein (arrow a) in HS5 membranes but not in C13/8

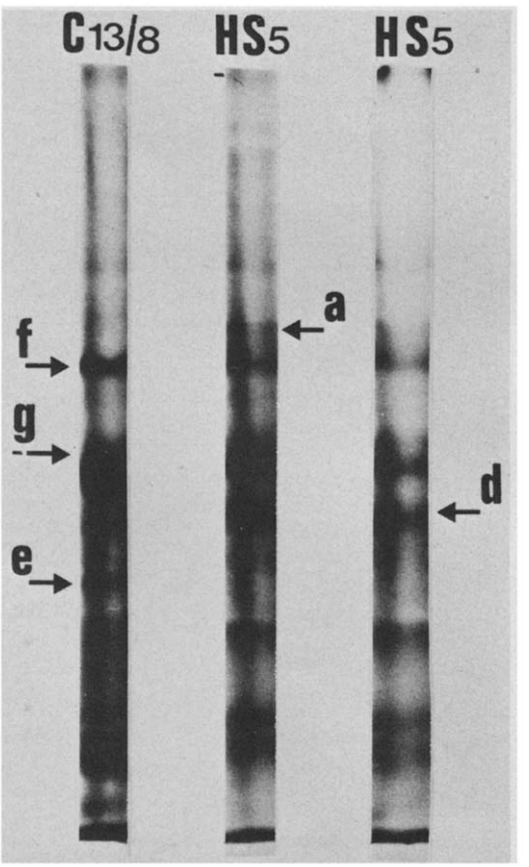
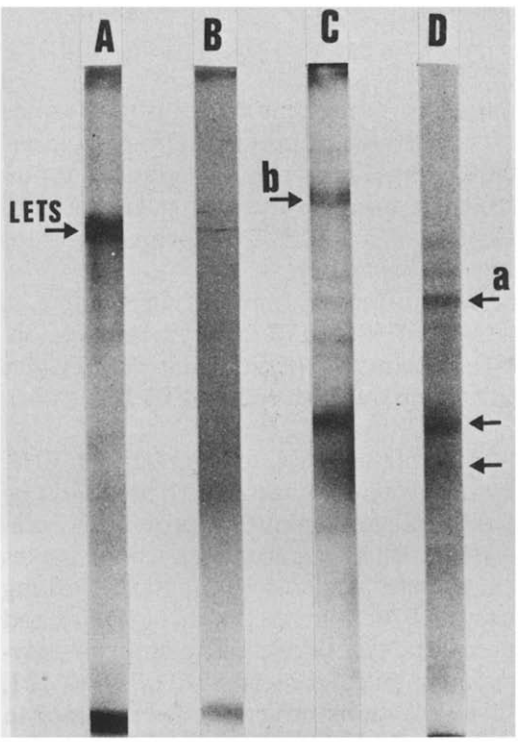


Fig. 6. Glucosamine-containing plasma membrane proteins. The figure shows the autoradiograms of plasma membrane fractions isolated from untransformed (C13/8) and transformed (HS5) cells which had been metabolically labelled with [^{14}C]glucosamine. See text for the meaning of arrows. Two lanes of the same HS5 autoradiogram developed after different exposition times are shown.

membranes. A second glucosamine labelled band (arrow d) is also increased in transformed cell membranes. Its position corresponds to M_r = 100 000. Third, a glucosamine band showing an apparent molecular weight of about 88 000 (arrow e) in untransformed membranes seems to be absent from transformed cell membranes. Other two major glycosylated bands (M_r = 160 000 and 120 000; arrow f and g) are present in membranes from both kinds of cells; these proteins can also be heavily labelled with [^{14}C]fucose (data not shown).

Iodinatable proteins in normal and transformed BHK cells

As can be seen in Fig. 7 (gels A and B), the major difference between C13/8 and HS5 cells in iodinated whole cells electrophoregrams was the absence in the transformed clone of the major iodinatable protein (arrow) showing an apparent molecular weight of 240 000. This transformation-sensitive protein, usually called LETS-protein, has been extensively studied by several laboratories [14]. This band appeared in our gels as a doublet with a secondary band at M_r = 230 000. Similar cell samples were treated with 10 μg/ml of trypsin for 10 min after iodination and then electrophoresed. The resulting gels were



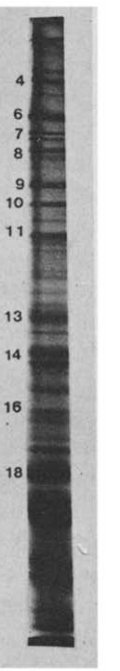


Fig. 7. Proteins accessible to enzymatic radioiodination. Autoradiograms of polyacrylamide gels of whole iodinated cells (lanes A and B) and of purified plasma membranes isolated from iodinated cells (lanes C and D). Material from untransformed (lanes A and C) and transformed (lanes B and D) cell cultures are compared. See text for the meaning of arrows.

Fig. 8. A provisional nomenclature for the major plasma membrane proteins of HS5 cells (taken from a typical electrophoretic pattern).

completely devoid of radioactivity bands showing that most, if not all proteins iodinated under these conditions were externally exposed, trypsin-sensitive, proteins. In subsequent experiments iodinated cells were used for plasma membrane purification. In both kinds of cells, in the final step of plasma membrane purification (flotation density gradient, see ref. 2) the radioactivity peak comigrates with the plasma membrane peak. In BHK cells 10% of initial trichloroacetic acid-insoluble counts could be recovered in the final membrane fraction with a 12-fold increase in specific radioactivity. Fig. 7 (gels C and D) shows the electrophoretic patterns of iodinated proteins from plasma membrane fraction. Besides the 240 000 transformation-sensitive double band (arrow b) 6—7 discrete iodinated bands could be identified in autoradiograms. Another major iodinated band was present in the position of the 177 000 transformation-increased protein (arrow a). Intensity of label in this band was also higher in transformed than in normal cells. Two other iodinated bands (arrows) were present at positions corresponding to M_r = 120 000 and 100 000, respectively. All these bands could also be identified on whole cell electrophoregrams with a long exposure time. The rest of the identifiable iodinated bands were not sufficiently labelled to allow further analysis.

Discussion

Provisional nomenclature

Electrophoretic analysis of plasma membrane fractions from untransformed and virus-transformed cells revealed a set of 18 major polypeptides of nominal molecular weight from 300 000 to 50 000 whose presence and relative proportions were highly reproducible. An arbitrary nomenclature of these bands is needed to simplify discussion. It is established in Fig. 8. This nomenclature is consistent with the one used in the preceding paper [2].

The results can be considered from two different points of view. First, in terms of the molecular characterization of the 177 000 transformation-increased protein (Band 10), and second, in terms of the information they give about other plasma membrane proteins in virus-transformed BHK cells. Both views will be discussed separately.

Comparing freeze-fractured plasma membrane from untransformed BHK cells, a 2—3-fold increase in the density of intramembranous particles could be seen [1]. This finding pointed to changes in integral membrane proteins accompanying viral transformation in this system, the magnitude of which makes them experimentally accessible with the present available techniques. Working on this possibility we have recently described the increase, in this transformed clone, of a large, presumably integral membrane glycoprotein showing, on polyacrylamide gel electrophoresis, an apparent molecular weight of 177 000 [2]. We have found a similar change in other virus-transformed clones and also in spontaneous BHK transformants (unpublished results). The data reported in this paper allow a closer characterization of this large, transformation-increased protein.

First, it is a product of cell metabolism because it can be metabolically labelled with [^{35}S]methionine.

Second, it is a membrane protein because it copurifies with morphologically

characterized plasma membrane fractions and with external iodine label.

Third, it is probably a glycoprotein because a [^{14}C]glucosamine band comigrates with this protein in dodecyl sulphate-polyacrylamide gel electrophoresis. Glucosamine label at this position is also transformation increased.

Fourth, it is partially exposed because it can be iodinated from outside with the non-penetrating lactoperoxidase reaction and because this label is trypsin sensitive. The same increase in the transformed clone in the amount of this protein can be detected either on Coomassie Blue stained gels or in ^{125}I-autoradiograms, which suggests that the iodine label is bound to the same increased protein.

Fifth, it behaves as an integral membrane protein because it can only be solubilized in the presence of detergents and SH-reducing agents. Once solubilized, it sediments in density gradients together with the whole set of proteins whose extraction depends upon detergent and mercaptoethanol.

In addition to the absolute requirement of mercaptoethanol for detergent solubilization, this protein can be cross-linked and erased from electrophoregrams by means of catalyzers of sulfhydryl oxidation. This results indicates that it is probably an SH-containing protein. Hynes and Destree [15] have recently pointed out that the surface of cultured cells has extensive disulfide bonding and that reducing agents improve extraction of several surface proteins.

Taking all these results as a whole, a provisional picture of the 177 000 transformation-increased protein emerges. It would be an integral membrane glycoprotein, partially exposed outside. The hypothesis of a relation of the increase in the amount of this protein in the transformed clone and the increase in density of intramembranous particles remains to be experimentally tested.

Besides the particular characterization of the transformation-increased membrane protein, the same set of experiments provide some data about several other membrane proteins of untransformed and HaSV-transformed BHK cells. We limited ourselves to the study of high molecular weight proteins which are better resolved in our electrophoretic conditions. It has been pointed out that plasma membranes are enriched in high molecular weight polypeptides as compared with other intracellular membrane systems [16].

At least 18 major electrophoretic bands (see Fig. 8) are visible in normal and transformed BHK cells. In general, no other identification criterion that the apparent molecular weight in polyacrylamide gels can now be established for most of these polypeptides. However some among them can be individualized on the basis of particular properties. These are the following:

(1) Two proteins of molecular weight 230 000 and 240 000 were collectively denominated Band 7. The relationship between these two proteins is not clear but they are both KCL extractable and both completely resistant to oxidative cross-linking. These proteins in Coomassie Blue-stained gels have the same position as LETS in ^{125}I-autoradiograms but they are neither trypsin nor transformation sensitive. Furthermore, it has been shown [15] that LETS-protein exists in the form of disulfide complexes and disappears from electrophoregrams if reduction is omitted. As the two underlying 230 000 and 240 000 Coomassie stained bands are not affected by oxidative cross-linking it is clear that they are not related to the LETS-protein.

(2) On the other hand, it should be pointed out that LETS-protein appeared always in ^{125}I-autoradiograms as a doublet (M_r = 230 000 and 240 000). Both iodinated bands were trypsin and transformation sensitive. The possibility of the existence of two different monomers of LETS-protein has been previously considered [15].

(3) Band 9 is a major non-glycosylated polypeptide (M_r = 195 000) which cannot be solubilized with any of the solution tested. This band is highly sensitive to oxidative cross-linking and its slightly decreased in transformed cells. It is not exclusively a membrane protein because it is also a major band in whole cell electrophoregrams where the rest of the major membrane bands are highly diluted. It is tempting to speculate a structural role for this protein.

(4) Band 5 (M_r = 275 000) calls our attention because of its high sensitivity to oxidative cross-linking, disappearing from electrophoregrams of oxidized samples together with Bands 9. Bands 4, 6 and 8 are also rapidly erased from cross-linked samples.

(5) Band 10 is the integral transformation-increased 177 000 protein whose properties have been just discussed.

(6) Band 11 is a 160 000 integral protein. The main glucosamine and fucose-containing band is found also at this position. Band 11 is also sensitive to cross-linking, but to a lesser extent, disappearing from cross-linked samples simultaneously with Band 10 but later than the other previously mentioned cross-link-sensitive bands.

(7) Band 13 is the second iodinatable band (M_r = 120 000) in membranes from both kinds of cells. This band is also a concanavalin-binding glycoprotein, which contains fucose and glucosamine. It cannot be solubilized either with low ionic strength EDTA nor with concentrated KCl but it can be extracted with 1% Triton X-100 without reducing agents.

(8) Band 14 (M_r = 98 000) is another iodinatable protein whose remarkable property is to be sensitive to extracellular glucose concentration. When both, normal and transformed cells are cultured in glucose depletion (unpublished results) this band doubled, because of the appearance of a secondary band at M_r = 96 000. A similar phenomenon has been recently described by Pouysségur and coworkers for mouse [17] and chicken [18] fibroblasts. Band 14 should be equivalent in our system, to the glucose-regulated protein, GRP 95, first described by these authors.

(9) A possible equivalent of the second glucose-regulated protein, GRP 78 [18], is also found in our system with an apparent molecular weight of 77 000. Both glucose-regulated proteins can be solubilized with Triton X-100 without reducing agents.

As previously proposed by Singer and Nicolson [19] membrane protein can be operationally classified as peripheral or integral, depending on whether they can be solubilized by chelating agents and salt solutions, or they need detergents to solubilize. Such different experimental behaviour is supposed to reflect different types of interaction with the lipid bilayer. In our system only Band 7 behaves as peripheral following those criteria. Perhaps several loosely bound peripheral proteins are lost during the long ghost isolation procedure. In the erythrocyte ghost, gentle isolation procedures allow the conservation in membrane fractions of loosely bound, salt-extractable proteins. Perhaps this

distinction is less applicable in the case of fibroblast ghosts purified through several steps after Zn-fixation.

We have also considered the possibility of using the requirement of reducing agents for detergent solubilization as another operational criterion for classifying the membrane proteins just described. However, here again, some artifactual associations between membrane proteins during the membrane purification procedure cannot be ruled out. Similar results must be obtained with several different membrane purification methods before the relevance of our data to membrane protein structure could be established.

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