Biochimica et Biophysica Acta, 551 (1979) 22—43

BBA 78267

AN ANALYSIS OF CONCANAVALIN A-MEDIATED AGGLUTINATION IN TWO CHINESE HAMSTER OVARY SUBCLONES WHOSE SURFACE PHENOTYPES RESPOND TO MAINTENANCE IN MEDIUM SUPPLEMENTED WITH DIBUTYRYL CYCLIC AMP

V. BIOCHEMICAL COMPOSITION OF THE PLASMA MEMBRANE

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(Received April 24th, 1978)
(Revised manuscript received September 6th, 1978)

Key words: Concanavalin A; Capping; Agglutination; (CHO clone)

Summary

We have used two Chinese hamster ovary subclones whose surface phenotype has been extensively investigated with regard concanavalin A-mediated cell-cell agglutination and concanavalin A-induced receptor site clustering to investigate what changes in membrane composition, if any, can be correlated with the concanavalin A-detected changes in surface phenotype. These cell clones are uniquely disposed for this purpose since maintenance of the cells under different growth conditions produces changes in agglutinability and receptor site mobility in one cell clone ($H\text{-}7_w$) but not the other (K-1). After extensive characterization of the surface membranes of these two subclones we have been unable to identify any change in the membrane peptides, glycopeptide, cholesterol, or fatty acid composition which can be directly correlated with the concanavalin A-detected surface phenotypes.

It is of particular interest to note that we have been unable to correlate the presence or absence of the large external transformation-sensitive glycoprotein with the relative mobility of the lectin receptors or with the degree of concanavalin A-mediated cell agglutination. Furthermore we have been unable, in this system, to corroborate earlier data suggesting a role for cholesterol in determining the relative mobility of the lectin receptors.

Thus using a cell system consisting of genetically matched cell clones, we have been unable to identify any changes in the biochemical composition of

Abbreviations: LETS protein, large external transformation-sensitive protein; PMSF, phenylmethylsulfonyl fluoride.

the plasma membrane which might be associated with the surface phenotypes detected by concanavalin A.

Introduction

Over the last few years many investigators have studied the binding of the lectin concanavalin A to the cell surface of lymphocytes and fibroblasts (for a review, see ref. 1). Most of these investigators have been interested in probing the molecular basis of concanavalin A-induced receptor site clustering or concanavalin A-induced agglutination of cells. A number of studies have provided data suggesting that surface components of the plasma membrane might be important in controlling both the mobility of concanavalin A receptors and the relative agglutinability of a cell line [2,3]. One set of data suggests that protease-labile surface components might be directly responsible for restricting both the relative agglutinability of a cell line and the relative mobility of the lectin receptors [2,3]. Other data suggest that changes in the relative fluidity of the plasma membrane, resulting from modifications in the phospholipid, fatty acid or cholesterol composition of the membrane, might be instrumental in determining the relative agglutinability and receptor site mobility of a particular cell type [4,5].

Despite the evidence which has accumulated suggesting that some change in the surface membrane might be important in determining the mobility of the concanavalin A receptors and/or the relative agglutinability of a cell line, no concerted effort has been made, to our knowledge, to identify changes in the composition of the plasma membrane which might be correlated with, if not responsible for, the surface phenotypes detected with concanavalin A. In previous work we have described a series of Chinese hamster ovary (CHO) cell clones which respond to growth in medium containing dibutyryl cyclic adenosine monophosphate by restricting the mobility of their concanavalin A receptors (Ellison, S., Mulholland, J. and Noonan, K.D., unpublished) as well as by reducing their relative agglutinability with concanavalin A [6]. Since these clones are genetically identical [22] and can be induced to rapidly modulate the surface phenotypes detected with concanavalin A (ref. 6 and Ellison, S., Mulholland, J. and Noonan, K.D., unpublished), we believe these subclones offer an ideal system in which to study surface changes which might be associated with concanavalin A-induced glycoprotein or glycolipid mobility or concanavalin A-mediated agglutinability.

Despite the advantages offered by these cell clones, the data we have accumulated strongly suggest that if differences in membrane structure exist between agglutinable and non-agglutinable cells, or between cells with mobile and non-mobile concanavalin A receptors, these differences in membrane composition must be relatively subtle and not immediately accessible to detection by standard membrane isolation and characterization techniques.

Materials and Methods

Materials. Concanavalin A was prepared from Jack bean meal according to the technique of Agrawal and Goldstein [7]. Fluorescein isothiocyanate and

dibutyryl cyclic adenosine monophosphate were purchased from Sigma Biochemicals (St. Louis, MO). All reagents used in electrophoretic analysis of plasma membrane peptides and glycopeptides were purchased from Bio-Rad Laboratories (Rockville Centre, NY). Tissue culture media and sera were obtained from Grand Island Biological Co. (Grand Island, NY). Tissue culture flasks and dishes were all purchased from Corning Glass Works (Corning, NY). All radioisotopes except ^{125}I were obtained from Amersham/Searle Corp. (Arlington Heights, IL). Scintillation cocktail (Phase Combining System) was also obtained from Amersham/Searle. ^{125}I was a product of Schwarz Mann Division of Becton, Dickinson and Co. (Orangeburg, NY). Neuraminidase was obtained from Worthington Biochemical Corp. (Freehold, NJ). Dextran 500 was obtained from Pharmacia (Piscataway, NJ). All reagents for electron microscopy were purchased from Polysciences, Inc. (Warrington, PA). All other laboratory reagents were purchased from Scientific Products (Ocala, FL).

Maintenance of the cell lines. Two subclones of CHO [22] were used throughout this work, K-1 and H-7_w. Both cell lines were maintained in McCoy's 5A growth medium supplemented with 10% (v/v) fetal calf serum. Cells were passaged every 2—3 days at approx. 80% confluency. The cells were tested for PPLO contamination by both autoradiography and nutrient agar [8,9] and found to be free of contamination.

Agglutination and lectin binding. All agglutinations were performed according to the techniques of Noonan and Burger [10]. Fluorescein tagged concanavalin A was prepared with fluorescein isothiocyanate as previously described (Ellison, S., Mulholland, J. and Noonan, K.D., unpublished). Capping of the concanavalin A receptors on the CHO cell surface was followed using a Wild M-20 microscope attached to a Wild transmitted light base III containing a ultraviolet light source and fitted with a dark field condensor. All photomicrographs were taken with a Nikormat FT2 camera attachment using Kodak Tri-X film.

Membrane isolation. Cells were removed from the tissue culture dish by washing the cells three times with calcium, magnesium-free phosphate-buffered saline (pH 7.2) containing 0.2% EDTA and 1 g/l glucose and then incubating the cells in this solution for 15 min at 37°C. Glucose was added to calcium, magnesium-free phosphate-buffered saline plus EDTA because we have found that it increased the rate at which cells were released from the plate and enhanced the relative viability of the cells released.

Following release from the tissue culture dish the cells were washed three times with phosphate-buffered saline (pH 7.2) and then a membrane fraction enriched in plasma membrane was isolated via the aqueous two-phase polymer system described by Brunette and Till [11]. Membranes were banded at least three times at the interface between the two polymer systems and then washed two times with phosphate-buffered saline to remove excess polymer. In some cases the final membrane pellet was washed with 1 mM Tris-HCl plus 1 mM EDTA (pH 7.5) in order to remove excess Zn^{2+} [12]. This final wash was particularly important in those experiments in which recovery of an enzyme activity was followed.

SDS-polyacrylamide gel electrophoresis. Electrophoretic separation of membrane peptides and glycopeptides was performed using the discontinuous gel

system described by Laemmli [13]. Our slab gels consisted of a linear gradient of 7.5—12.5% acrylamide overlayed with a 5.6% stacking gel. This electrophoretic system efficiently separated membrane components between ~300 000 and ~15 000 molecular weight. The acrylamide to bis-acrylamide ratio was maintained at 37.5 : 1 in both the stacking and running gel. In those separations carried out in cylindrical tube gels, the technique of Fairbanks et al. [14] was employed using a 7.5% acrylamide running gel. In order to quantitate radiolabel incorporation into membrane peptides, tube gels containing leucine-labeled peptides were frozen, sliced into 2-mm sections, solubilized in H_2O_2 and counted in Phase Combining System (Amersham-Searle). Both the slab gels and tube gels were stained with Coomassie Brilliant Blue and destained according to the technique of Weber and Osborn [15].

RNA, DNA and protein determinations. RNA was determined using the acid-orcinol technique [16]. DNA was determined according to the technique of Burton [17]. Protein concentration was determined via the procedure of Lowry et al. [18].

Enzyme assay. ($Na^+ + K^+$)-activated ATPase was measured according to the modifications described by Brunette and Till [11]. Glucose-6-phosphatase was measured according to the technique described by Franke et al. [19]. Inorganic phosphate release in both experiments was measured via a modification of the Fiske-Subbarow technique [20].

Electron microscopy. Membrane samples were fixed for 1 h at 4°C in 2% glutaraldehyde and then dehydrated through successively increasing concentrations of ethanol. Fixed samples were embedded in Spurr's low viscosity medium [21] and sectioned. Silver sections were post-stained with uranyl acetate and examined with a Siemens Elmiskop 101 at 80 kV.

Phospholipids, fatty acids and cholesterol. Phospholipids, fatty acids and cholesterol were determined from a chloroform/methanol extract of the cell fractions. Lipid phosphorus was determined according to the method of Chen et al. [20]. A factor of 25 was used to convert μg phosphorus to μg phospholipid. Cholesterol was measured by the procedure of Glick et al. [24].

Labeling of membrane components. Membrane peptides were metabolically labeled by maintaining cells for 48 h in medium containing 2.5 μCi/ml L-[4,5-^{3}H]leucine or 0.625 μCi/ml L-[1-^{14}C]leucine. Glycopeptides were metabolically labeled by maintaining cells in medium supplemented with 2.5 μCi/ml D-[1-^{3}H]glucosamine hydrochloride. Following electrophoretic separation of membrane peptides and glycopeptides, the concanavalin A binding glycoproteins were labeled in the acrylamide slab gel with ^{125}I-labeled concanavalin A via a modification of the technique described by Burridge [25]. The concanavalin A binding glycoproteins were displayed by autoradiography of the dried gel.

Labeling of the cells with ^{125}I or NaB^3H_4 was performed after the cells had been removed from the tissue culture plate and washed three times with phosphate-buffered saline as described in the section on membrane isolation. Peptides and glycopeptides were labeled with 400 μCi/ml ^{125}I according to the lactoperoxidase technique of Phillips and Morrison [26]. Sialic acid residues were labeled with $NaIO_4/NaB^3H_4$ by incubating cells in calcium, magnesium-free phosphate-buffered saline (pH 7.4) containing 1 mM $NaIO_4$ for 20 min at

0° C. Following this, the cells were again washed twice with phosphate-buffered saline and then incubated 20 min at room temperature in phosphate-buffered saline containing 1.25 mCi/ml NaB^3H_4. The cells were finally washed two more times with phosphate-buffered saline and then the membranes isolated. Galactose residues were labeled with NaB^3H_4 using the galactose oxidase technique described by Critchley [28].

[^{32}P]Orthophosphate incorporation into membrane proteins. H-7_w and K-1 cells were maintained for 48 h in the presence or absence of 1 mM dibutyryl cyclic AMP plus 2.5 μCi/ml [^{32}P]orthophosphate. Membranes were isolated as described, washed three times with phosphate-buffered saline and then incubated for 1 h at 37° C with 100 μg/ml RNAase A and 100 μg/ml DNAase I which had been demonstrated to be protease free via the technique of Tomarelli et al. [45]. Following nuclease digestion the membranes were again washed with phosphate-buffered saline and then the phospholipids were extracted with chloroform/methanol. Membrane proteins were recovered from the interface of the polar and non-polar phases, solubilized in Laemmli 'dialysis buffer' and applied to a 7.5—12.5% SDS slab gel. The gels were stained with Coomassie Brilliant Blue as described and an autoradiograph of the slab prepared.

Antibody precipitation of LETS protein. 10 μl of rabbit antiserum raised against SDS gel-purified LETS protein [38] (kindly provided by Dr. H.O. Hynes, Massachusetts Institute of Technology) was added to 1.0 ml of supernatant derived from control or trypsin-treated, ^{125}I-labeled cells. Both the control and trypsinized supernatants were brought to 1 mM PMSF to prevent or eliminate proteolytic activity. 10 μl of pre-immune serum was added to the supernatants derived from control and trypsin-treated cells in order to determine the specificity of the immune precipitation. With both the immune and pre-immune sera the supernatants were incubated 24 h at 4° C. 100 μl of goat anti-rabbit antiserum was then added to the supernatants from all the samples and incubated for 6 h at 4° C. Following incubation the samples were centrifuged at 1500 × *g* for 15 min and the precipitates collected. Pellets were washed three times with phosphate-buffered saline and then solubilized as for separation on SDS gels. Aliquots were taken from each sample and the counts released determined.

Autoradiography and fluorography. The relative mobility of iodinated membrane components on SDS slab gels was determined by autoradiography of dried slab gels. The distribution of ^{3}H-labeled membrane components on slab gels was displayed via the fluorographic technique of Bonner and Laskey [29].

Results

As we have previously demonstrated [6], growth of H-7_w and K-1 cells in medium containing 1 mM dibutyryl cyclic AMP produces a shape change in these two subclones which has been described as a change from an 'epithelioid' to 'fibroblastic' morphology (see ref. 30 for an extensive scanning electron microscopy study of this morphologic change). We have also demonstrated that maintenance of H-7_w cells in medium containing 1 mM dibutyryl cyclic AMP significantly reduces the relative agglutinability of this subclone with

concanavalin A while maintenance of the K-1 subclone in medium containing 1 mM dibutyryl cyclic AMP has no apparent effect on the relative agglutinability of that subclone [6] (Table I). Maintenance of the H-7_w subclone in medium containing the cyclic nucleotide analogue produces a surface phenotype in which fl-concanavalin A bound to the cell surface retains a random distribution (Eillison, S., Mulholland, J. and Noonan, K.D., unpublished) (Fig. 1D). On the other hand, fl-concanavalin A bound to H-7_w or K-1 cells maintained in the absence of dibutyryl cyclic AMP (Figs. 1A and 1C), as well as fl-concanavalin A bound to K-1 cells maintained in medium containing 1 mM dibutyryl cyclic AMP (Fig. 1B), localizes to a 'cap' within 15 min after the addition of the lectin to the cells (Ellison, S., Mulholland, J. and Noonan, K.D., unpublished). Since the K-1 subclone differs from the H-7_w subclone in its apparent lack of a concanavalin A-detectable response to maintenance in 1 mM dibutyryl cyclic AMP (as defined by the data in Table I and Fig. 1, as well as ref. 6 and (Ellison, S., Mulholland, J. and Noonan, K.D., unpublished), we felt that characterization of the surface composition of H-7_w and K-1 cells maintained in the presence and absence of dibutyryl cyclic AMP might allow us to identify changes in membrane composition correlated with the control of concanavalin A-induced receptor site mobility and/or agglutinability.

Membrane isolation and relative purity

Cells were removed from the tissue culture dish by repeated washings with calcium, magnesium-free, phosphate-buffered saline containing EDTA and glucose as described in Materials and Methods. Care was taken during the washing and incubation steps prior to membrane isolation to maintain the appropriate cells in buffer containing dibutyryl cyclic AMP. A membrane fraction was isolated by the two-phase aqueous polymer technique of Brunette and Till [11] and subsequently washed in either phosphate-buffered saline or Tris-EDTA as described in Materials and Methods. The yield of membrane protein isolated via this technique is approx. 5.5% of the total cell protein.

Fig. 2 is an electron micrograph of the membrane pellet obtained from H-7_w cells. The isolated fraction is clearly composed of sheets of membrane which are free of any obvious contamination with other cellular organelles. It should be noted, however, that a granular material is associated with the membrane

TABLE I

CONCANAVALIN A AGGLUTINATION OF H-7_w AND K-1 SUBCLONE MAINTAINED IN THE PRESENCE OR ABSENCE OF DIBUTYRYL CYCLIC AMP

All agglutinations were performed at 30°C with 30 μg/ml concanavalin A according to the technique described by Noonan and Burger [10]. Preincubation of the lectin with 1 mM α-methyl mannose reduced the agglutinability of the cells to less than 10% of the control.

Subclone	Agglutination (%)	
	Minus 1 mM dibutyryl cyclic AMP	Plus 1 mM dibutyryl cyclic AMP
H-7_w	95	10
K-1	95	95

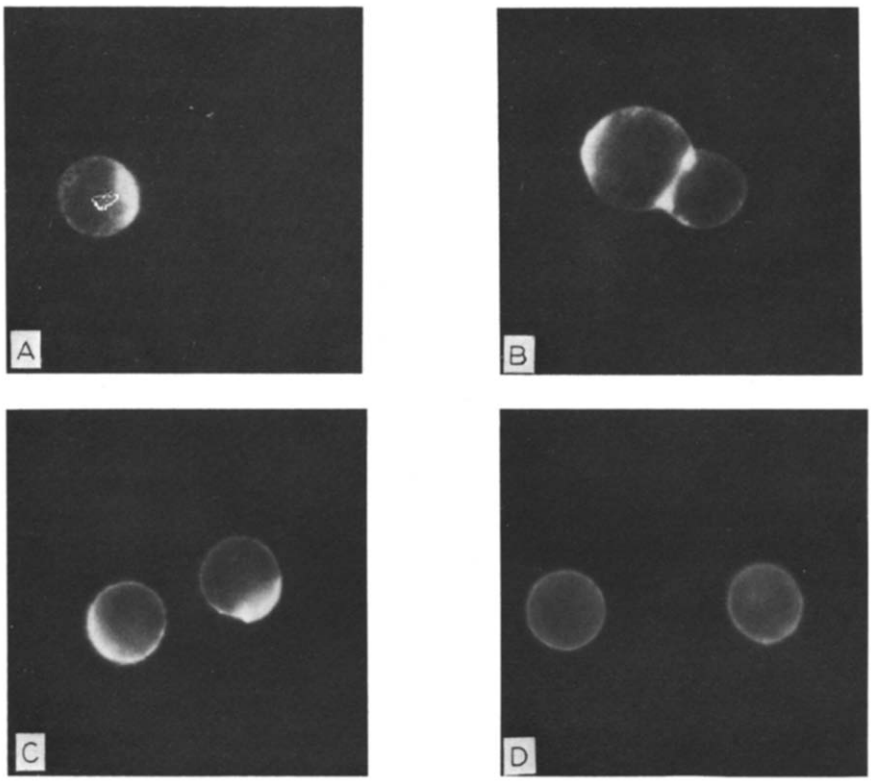

Fig. 1. fl-concanavalin A distribution on CHO cell surface. H-7_w and K-1 cells were maintained for 48 h in medium with or without 1 mM dibutyryl cyclic AMP. The cells were then removed from the substratum according to the technique of Noonan and Burger [10]. Cells were incubated 15 min at 30° C with 30 μg/ml fl-concanavalin A, chilled to 0° C and the distribution of fl-concanavalin A determined. (A) K-1 cells maintained in the absence of dibutyryl cyclic AMP. (B) K-1 cells maintained in the presence of dibutyryl cyclic AMP. (C) H-7_w cells maintained in the absence of dibutyryl cyclic AMP. (D) H-7_w cells maintained in the presence of dibutyryl cyclic AMP.

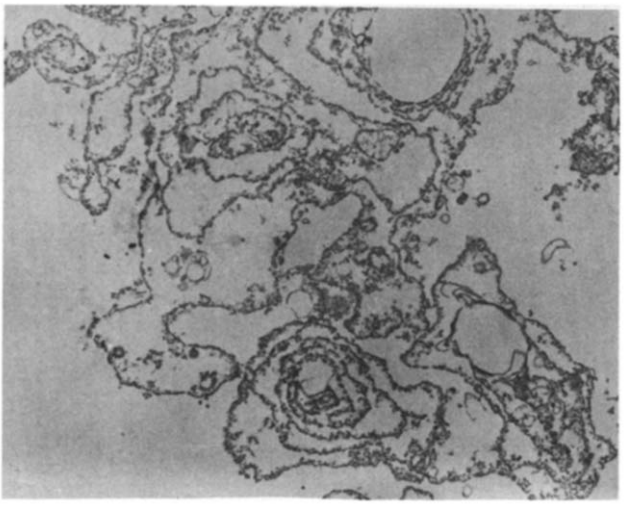

Fig. 2. Electron micrograph of H-7_w membrane pellet. Membrane pellet was prepared as described in Materials and Methods (×5000).

sheets. This granular material has been seen in most membranes isolated via techniques which rely on $ZnCl_2$ to 'tan' the cells prior to homogenization and probably represents adherent cytoplasmic material.

Table II displays the specific activity of a number of purported 'markers' of the plasma membrane. The data in Table II relate only to the H-7_w subclone grown in the presence and absence of dibutyryl cyclic AMP, however essentially the same data has been derived from the membranous pellet obtained from K-1 cells maintained in the presence or absence of the nucleotide analogue. ($Na^+ + K^+$)-ATPase, which has frequently been used as a marker for plasma membrane, is approx. 20-fold enriched in the membrane pellet as compared to the homogenate. Iodination of the surface membrane prior to membrane isolation results in an approx. 4-fold enrichment of iodinated material in the membranous fraction as compared to the homogenate. Using cholesterol as a relatively non-specific marker of plasma membrane, we have found that the membrane-enriched fraction obtained from the two-phase membrane preparation displays a 4-fold enrichment relative to the homogenate. Using $NaIO_4$/NaB^3H_4 labeling of the cells to label sialic acid residues (frequently used as a cell surface marker [12]) prior to homogenization and membrane isolation, we find that the final membrane-enriched fraction shows a 6.5-fold increase in $NaIO_4/NaB^3H_4$-labeled material as compared to the homogenate. Thus, depending on which marker one chooses, the data obtained suggests that a 4—20-fold enrichment of plasma membrane is obtained in the membranous pellet relative to the homogenate. These data are comparable to data previously published by Juliano and Behor-Bannelier [23] with regard to the purity of CHO membranes isolated via two-phase technique.

Table II also demonstrates that the membrane fraction isolated by the two-phase aqueous polymer system is relatively free of DNA contamination while there is an enrichment of RNA in the membrane fraction relative to the homogenate. Whether this RNA is soluble, cytoplasmic RNA which becomes associated with the membrane during isolation or whether it represents a contamination of the membranous fraction with microsomes is not certain. That we are not specifically enriching for endoplasmic reticulum in the isolation procedure is suggested by the electron micrograph (Fig. 2) which fails to show structures which are immediately identifiable as ribosomes and by the fact that the specific activity of glucose-6-phosphatase (a purported marker of microsomal membranes [27]) is approx. 1.5-fold lower in the membranous fraction as compared to the homogenate (Table II). On the basis of all the accumulated data (Fig. 2 and Table II) we conclude that we are working with a membranous fraction which is predominantly plasma membrane but which is contaminated with exogenous RNA from an unidentified source. We do not feel, however, that the RNA associated with the membranous pellet invalidates the data presented in this manuscript.

Peptide composition

Fig. 3 displays the Coomassie Brilliant Blue staining profile of the peptides and glycopeptides of the membrane fraction isolated from H-7_w and K-1 cells maintained in the presence or absence of 1 mM dibutyryl cyclic AMP. As can be seen, there are no obvious qualitative differences in the Coomassie Brilliant

TABLE II

SPECIFIC ACTIVITY OF PLASMA MEMBRANE MARKERS

$(Na^+ + K^+)$-activated ATPase was assayed according to the technique outlined by Brunette and Till [11]. Surface membranes were labeled with ^{125}I via the technique discussed in Materials and Methods. Surface membranes were labeled with $NaIO_4/NaB^3H_4$ as discussed in Materials and Methods. Cholesterol was determined via the technique of Glick et al. [34]. DNA was measured using the technique of Burton [17] while RNA contamination was determined by the acid-orcinol procedure [16]. Glucose-6-phosphatase activity was determined according to the technique of Franke et al. [19]. N.D., not determined.

	$(Na^+ + K^+)$-ATPase (μmol P_i/mg protein per h)	Glucose-6-phosphatase (μmol P_i/mg protein per h)	^{125}I (cpm/mg protein)	$NaIO_4NaB^3H_4$ (cpm/mg protein)	Cholesterol (μg/mg protein)	DNA (μg/mg protein)	DNA (μg/mg protein)
Homogenate							
$H\text{-}7_W$	0.40	0.59	1464	1307	50.5	95.9	99.4
$H\text{-}7_W$ + dibutyryl cyclic AMP	0.44	N.D.	967	1195	57.8	138.6	82.3
Membrane fraction							
$H\text{-}7_W$	7.81	0.38	6451	10 156	193.0	28.1	177.5
$H\text{-}7_W$ + dibutyryl cyclic AMP	8.21	N.D.	6275	6031	197.8	25.1	149.6

N.D., not determined.

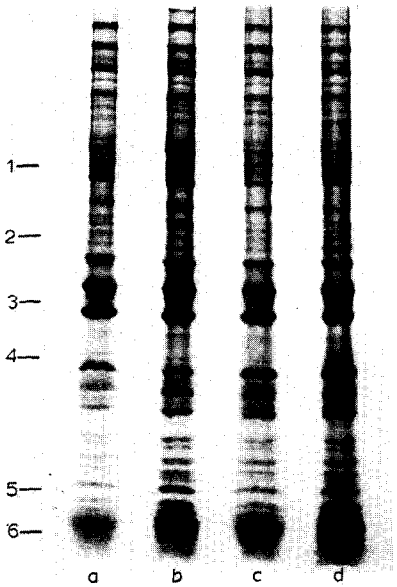

Fig. 3. Coomassie Brilliant Blue staining profile of CHO membrane fractions. H-7_w and K-1 cells were maintained for 48 h in medium with or without 1 mM dibutyryl cyclic AMP. After isolation the membrane fractions were solubilized according to Laemmli [13]. 50 μg of membrane protein were applied to each gel slot and separated according to Laemmli [13] using a 5.6% acrylamide stacking gel overlying a 7.5—12.5% linear acrylamide gradient. After separation the membrane peptides and glycopeptides were stained with Coomassie Brilliant Blue and destained according to the technique of Weber and Osborn [15]. From above to below lines representing molecular weight markers: 1, phosphorylase *a* (100 000); 2, bovine serum albumin (67 000); 3, ovalbumin (43 000); 4, DNAase I (31 000); 5, soybean trypsin inhibitor (21 500); 6, cytochrome *c* (12 400). From left to right: a, H-7_w; b, H-7_w + dibutyryl cyclic AMP; c, K-1; d, K-1 + dibutyryl cyclic AMP.

Blue staining profile derived from the membranes isolated from the four separate experimental populations. It is worth noting the extreme complexity of the patterns observed in each of the membrane fractions. Such a complexity could, of course, hide subtle differences in peptide and glycopeptide composition which might exist among the different membrane isolates.

Fig. 4A is a densitometric scan of H-7_w membrane peptides separated on a 7.5% acrylamide gel according to the technique of Fairbanks et al. [14]. The densitometric scans of membrane peptides isolated from H-7_w cells maintained in medium containing 1 mM dibutyryl cyclic AMP as well as K-1 cells maintained in medium with or without dibutyryl cyclic AMP are superimposable on the profile presented in Fig. 4A. Figs. 4B and 4C display the results of a dual radiolabel experiment directed at detecting any quantitative differences in the peptide composition of H-7_w or K-1 cells maintained in the presence or absence of dibutyryl cyclic AMP. Again, no striking differences are observed among the profiles obtained from the four membrane preparations.

Identification of concanavalin A binding glycoproteins

Fig. 5 is an autoradiogram displaying the number and apparent molecular weights of the concanavalin A binding glycopeptides which are present in H-7_w cell membranes isolated from cells maintained in the presence or absence of

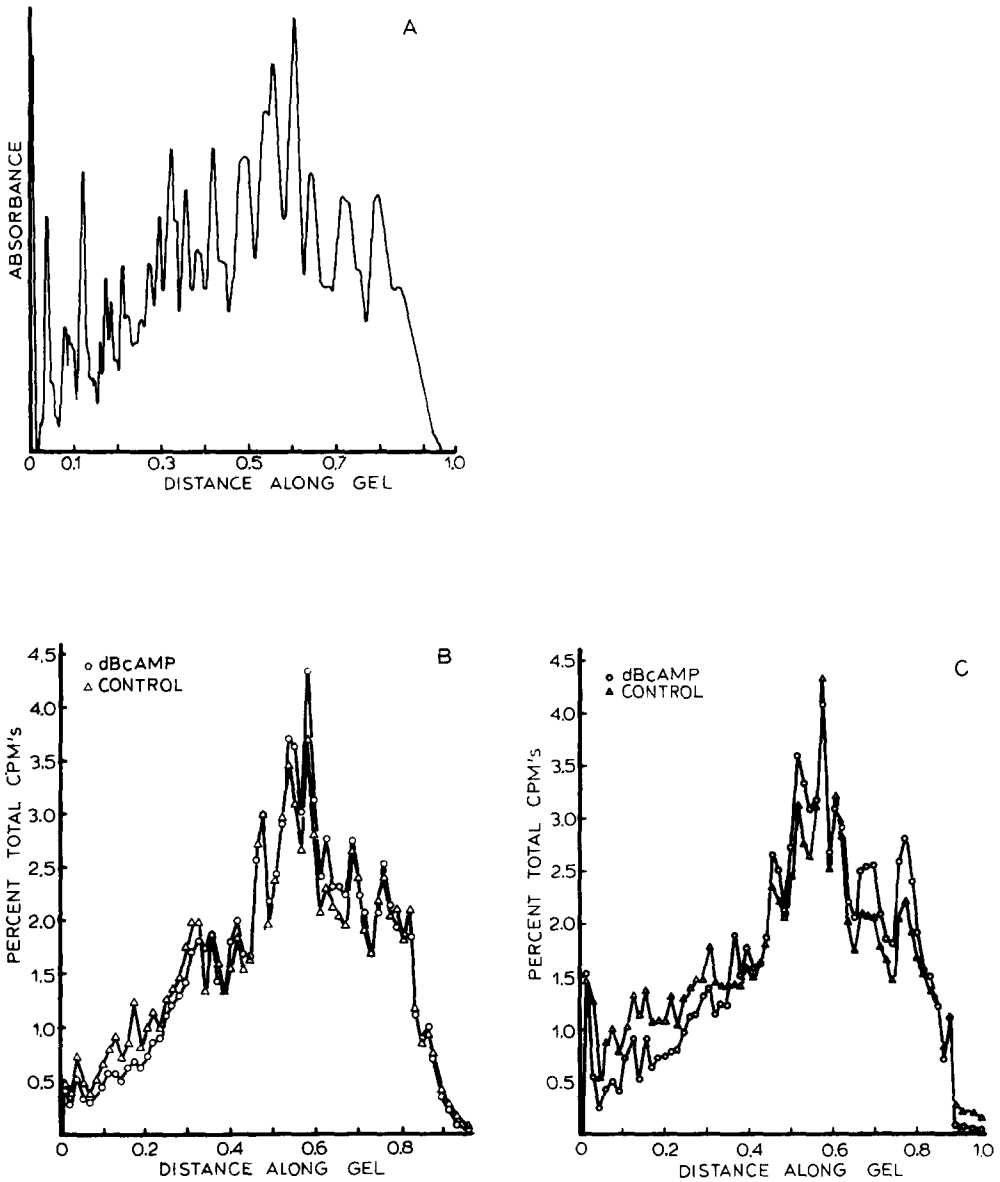

Fig. 4. Double labeling H-7$_W$ and K-1 membranes. H-7$_W$ and K-1 cells were maintained 48 h in medium with and without 1 mM dibutyryl cyclic AMP containing 2.5 μCi/ml L-[4,5-^{3}H]leucine (+dibutyryl cyclic AMP) or 0.625 μCi/ml L-[1-^{14}C]leucine (control). Membranes were isolated from the four separate experiments. 50 μg [^{3}H]leucine and 50 μg [^{14}C]leucine-labeled membranes were mixed and then applied to a 7.5% acrylamide gel and the components separated according to Fairbanks et al. [14]. After separation the gels were stained and destained according to Weber and Osborn [15]. After scanning, the gels were frozen, sliced into 2-mm sections, solubilized overnight in H_2O_2 and finally counted in Phase Combining System (PCS) (Amersham/Searle). (A) Densitometric scan of H-7$_W$ membranes separated on a 7.5% acrylamide gel and stained with Coomassie Brilliant Blue. (B) H-7$_W$ with and without dibutyryl cyclic AMP (dBcAMP). Plot of [^{3}H]- and [^{14}C]leucine present in each gel slice. ^{3}H and ^{14}C counts were normalized by determining the percent total counts in each slice. ~50 000 cpm [^{3}H]leucine and ~30 000 cpm [^{14}C]leucine was applied to the gel. (C) K-1 with and without dibutyryl cyclic AMP (dBcAMP). Plot of [^{3}H]- and [^{14}C]leucine present in each gel slice. ^{3}H and ^{14}C counts were normalized by determining the percent total counts in each slice. ~50 000 cpm [^{3}H]leucine and ~30 000 cpm [^{14}C]leucine were applied to the gel.

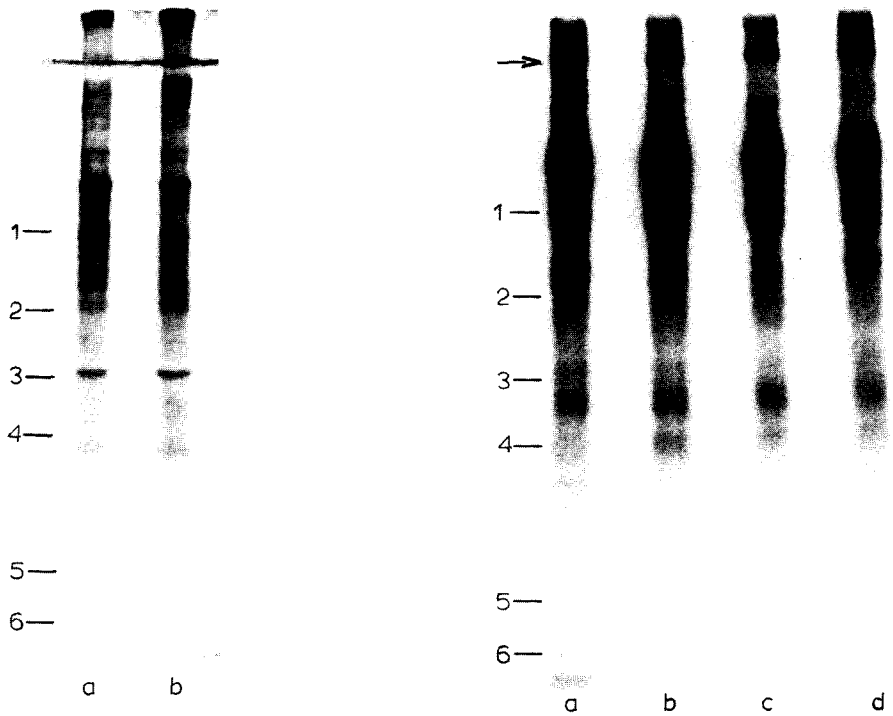

Fig. 5. Concanavalin A binding glycopeptides in H-7_w membrane fractions. H-7_w cells were maintained 48 h with and without dibutyryl cyclic AMP. Membrane fractions were isolated, the membranes solubilized according to Laemmli [13] and 50 μg protein applied to a gradient slab gel. After separation the gel was stained and destained as described in Fig. 3 and then overlayed with ^{125}I-labeled concanavalin A via a slight modification of the technique described by Burridge [25]. The gel was finally dried and an autoradiogram produced. Incubation of ^{125}I concanavalin A with α-methyl mannose prior to the addition of the lectin to the gel resulted in no distinct concanavalin A binding bands appearing in the autoradiogram. Molecular weight markers (1—6) as in Fig. 3. a, H-7_w; b, H-7_w + dibutyryl cyclic AMP.

Fig. 6. Membrane components which could be iodinated. H-7_w and K-1 cells were maintained for 48 h in medium with and without dibutyryl cyclic AMP. Cells were iodinated as described in Materials and Methods and then a membrane fraction isolated. The membrane fraction was solubilized in SDS and applied to the gradient gel described in Fig. 3. Approx. 100 000 cpm were applied to each gel slot. After separation the gel was dried and an autoradiogram prepared. Molecular weight markers (1—6) as in Fig. 3. a, H-7_w; b, H-7_w + dibutyryl cyclic AMP; c, K-1 and d, K-1 + dibutyryl cyclic AMP. An arrow indicates the band tentatively identified as the LETS protein [36].

dibutyryl cyclic AMP. Two points which strike one with regard to Fig. 5 are the heterogeneity of the concanavalin A receptors in terms of numbers and apparent molecular weights (20 receptors ranging in molecular weight from ~220 000 to 25 000) and the lack of any discernible quantitative or qualitative differences in the concanavalin A receptors displayed by the H-7_w cells maintained in the presence or absence of dibutyryl cyclic AMP. K-1 cells maintained in the presence or absence of dibutyryl cyclic AMP display the same concanavalin A binding glycopeptides.

This finding that there are no apparent differences in the number of different concanavalin A receptors on the H-7_w or K-1 cell membranes following maintenance of the cells in 1 mM dibutyryl cyclic AMP is supportive of our

previous data [6] demonstrating that both cell clones (under both growth conditions) displayed essentially the same number of concanavalin A receptors.

Surface peptides and glycopeptides

Fig. 6 is an autoradiograph on which the membrane components which could be iodinated are distributed. As with the concanavalin A binding glycoproteins, an extreme heterogeneity exists among the surface components of these CHO cells which could be iodinated. From this autoradiograph it can be seen that there are two major 'areas' on the gel containing iodinated material along with many discrete bands outside of these two areas. One 'area' is distributed between molecular weights 185 000 and 150 000 while the other major 'area' runs between molecular weights 110 000 and 90 000. In autoradiographs exposed for shorter periods of time, it is evident that these 'areas' are composed of a number of discrete iodinated species. No obvious differences in components which could be iodinated are detected among the four samples analyzed. Separating the iodinated components on gels of other acrylamide concentrations similarly failed to reveal any differences among the species of the four samples which could be iodinated. It is especially interesting to note that both $H\text{-}7_w$ and K-1 cells maintained in the presence or absence of dibutyryl cyclic AMP contain a membrane component which could be iodinated with an apparent molecular weight of ~260 000 (arrow). Since this is one of the major components of the membrane fraction which could be iodinated, is very trypsin labile (data not shown), and runs at an appropriate molecular weight, it is undoubtedly analogous to the so-called LETS protein which has been thoroughly investigated as a potential determinant of transformation as well as cell-cell and cell-substratum adhesion (see for example refs. 31–33).

Better evidence that this trypsin labile, iodinated surface protein is indeed the LETS protein is shown in Table III. As can be seen, treatment of iodinated $H\text{-}7_w$ cells with 10 μg/ml trypsin for 10 min releases approx. 55% of the iodinated surface components and that approx. 80% of the released, iodinated

TABLE III

IDENTIFICATION OF TRYPSIN-RELEASED MATERIAL AS LETS

$H\text{-}7_w$ cells were removed from the tissue culture plate as described in Materials and Methods. The cells were then labeled with ^{125}I according to the technique of Phillips and Morrison [26]. Following labeling, half of the cells were incubated in phosphate-buffered saline while the remaining cells were incubated for 10 min with 10 μg/ml trypsin at room temperature. Both aliquots were pelleted at 1000 rev./min and the supernatant collected and brought to 1 mM PMSF to inhibit proteolytic activity. The supernatant from each incubation was then again divided in half, one half receiving immune serum and the other pre-immune serum. The precipitation of LETS with immune sera was performed as described in Materials and Methods. Essentially the same results have been obtained with $H\text{-}7_w$ cells maintained in the presence of 1 mM dibutyryl cyclic AMP and with K-1 cells maintained in the presence or absence of 1 mM dibutyryl cyclic AMP.

	cpm/μg protein in isolated membranes	Total cpm released into supernatant	cpm precipitated by anti-LETS	cpm precipitated by pre-immune sera
Control	$5.7 \cdot 10^3$	$1.2 \cdot 10^3$	$1.9 \cdot 10^2$	$1.0 \cdot 10^2$
+ trypsin	$2.5 \cdot 10^3$	$6.6 \cdot 10^6$	$5.4 \cdot 10^6$	$5.0 \cdot 10^4$

material can be specifically precipitated by an antibody directed against the LETS glycoprotein. Pre-immune serum did not precipitate significant quantities of the iodinated material released by proteolysis from the H-7_w cell membrane. Essentially the same quantitative and qualitative data as presented in Table III was obtained when H-7_w cells maintained in 1 mM dibutyryl cyclic AMP or K-1 cells maintained in the presence or absence of 1 mM dibutyryl cyclic AMP were treated as described in Table III (data not shown).

Fig. 7 is a fluorograph displaying membrane glycoproteins which have been metabolically labeled with [^{3}H]glucosamine and then separated on a 7.5—12.5% gradient slab gel. As in the profile displayed by iodinated membrane components, glucosamine labels two major 'areas' of the gel (molecular weights

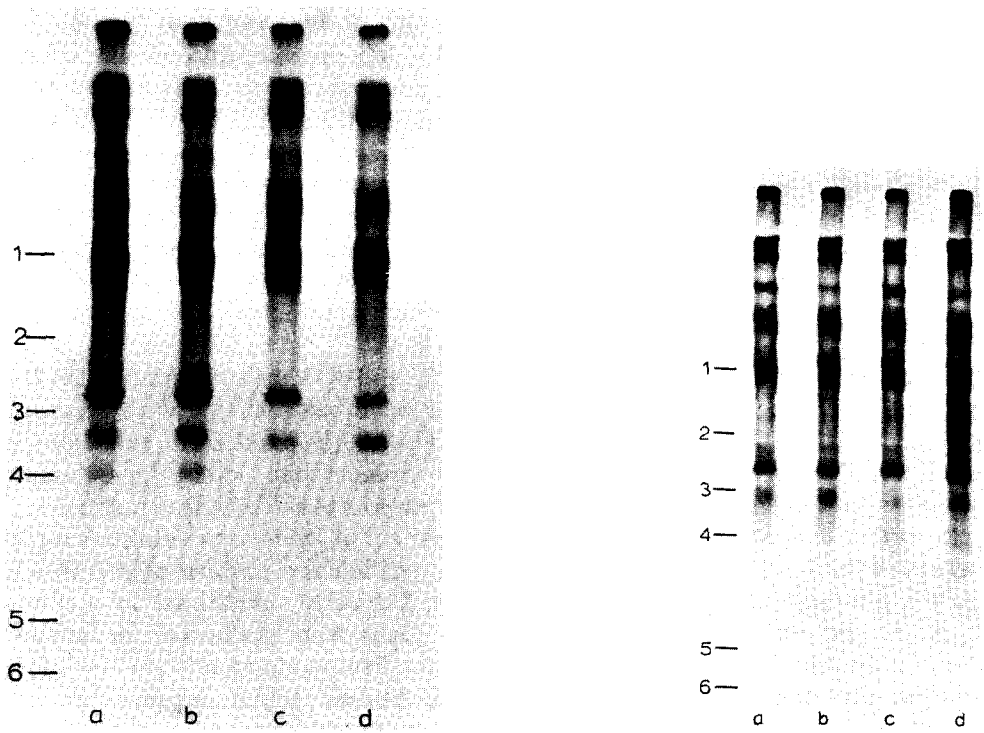

Fig. 7. [^{3}H]Glucosamine-labeled glycopeptides. H-7_w and K-1 cells were maintained for 48 h in media with and without dibutyryl cyclic AMP containing 2.5 μCi/ml D-[1-^{3}H]glucosamine hydrochloride. The membrane fractions were isolated, solubilized and equal number of counts applied to the gradient slab gel described in Fig. 3. After separation the gel was prepared for fluorography according to the technique of Bonner and Laskey [29]. Molecular weight markers (1—6) as in Fig. 3. a, H-7_w; b, H-7_w + dibutyryl cyclic AMP; c, K-1 and d, K-1 + dibutyryl cyclic AMP.

Fig. 8. $NaIO_4$/NaB^3H_4-labeled glycopeptides. H-7_w and K-1 cells were maintained for 48 h in media with and without dibutyryl cyclic AMP. The cells were then removed from the plate and labeled with $NaIO_4$/NaB^3H_4 as described in Materials and Methods. The membrane fractions were isolated, solubilized and equal numbers of counts (~100 000) were applied to the gradient gel described in Fig. 3. After separation the gel was prepared for fluorography according to the technique of Bonner and Laskey [29]. Molecular weight markers (1—6) as in Fig. 3. a, H-7_w; b, H-7_w + dibutyryl cyclic AMP; c, K-1 and d, K-1 + dibutyryl cyclic AMP. Incubation of $NaIO_4$/NaB^3H_4-labeled membranes with 10 μg/ml neuraminidase for 1 h at 37°C released 60% of the label from the membranes suggesting that the majority of the ^{3}H is associated with sialic acid residues.

~200 000—160 000 and 110 000—90 000). Many other 'lesser' bands appear in the fluorograph with three major bands appearing at apparent molecular weights of 260 000, 235 000 and 67 000. There is no evidence that qualitative or quantitative differences exist among the membrane glycopeptides isolated from the four samples. Distribution of the glucosamine-labeled components on gels of other acrylamide concentrations also failed to reveal any differences among the glucosamine-labeled components of the isolated CHO membranes.

Previous evidence from our laboratory suggested that the relative sialylation of a class of glycofucopeptides which could be released from the cell surface by limited proteolysis might be important in determining the relative agglutinability of the different subclones [34]. In order to further investigate this phenomenon, H-7_w and K-1 cells (maintained in the presence or absence of dibutyryl cyclic AMP) were labeled with $NaIO_4/NaB^3H_4$ to label sialic acid-containing glycopeptides among the four membrane samples. Fig. 8 is a fluorograph demonstrating that, under the labeling conditions employed, $NaIO_4/NaB^3H_4$ labels essentially the same components as [^{3}H]glucosamine. Two 'areas' at molecular weights 200 000—160 000 and 110 000—90 000, as well as major bands at 260 000 and 65 000, appear in the fluorograph (Fig. 8). $NaIO_4/NaB^3H_4$ also labels major bands which run with apparent molecular weights of 55 000 and 45 000, as well as other minor bands which distribute throughout the molecular weight range. Again no obvious differences appear among the four samples, suggesting that our earlier results could not be accounted for by one major protease-labile, sialofucopeptide present on agglutinable but not non-agglutinable cell surfaces.

Galactose residues on the surfaces of H-7_w and K-1 cells maintained in the presence or absence of dibutyryl cyclic AMP have been labeled with galactose oxidase/NaB^3H_4 [28]. Fluorographs of membranes isolated from cells labeled via this technique have essentially the same profile as that seen in Fig. 8. Again, no obvious differences appeared among the four samples (data not shown).

In order to determine whether maintenance of cells in medium supplemented with 1 mM dibutyryl cyclic AMP modified the pattern of membrane protein phosphorylation [39,40], H-7_w and K-1 cells maintained in medium supplemented with the cyclic nucleotide analogue were metabolically labelled with ^{32}P as described in Materials and Methods. Following membrane isolation, nuclease digestion (to remove contaminating nucleic acids) and phospholipid extraction, the membrane phosphoproteins were separated on a 7.5—12.5% SDS slab gel and then an autoradiograph prepared. Fig. 9A is the Coomassie Brilliant Blue-stained slab gel while Fig. 9B is the autoradiograph. As can be seen, maintenance of both the H-7_w and K-1 cells in medium supplemented with 1 mM dibutyryl cyclic AMP did induce the phosphorylation of two membrane proteins (arrows) which were not phosphorylated in the absence of dibutyryl cyclic AMP. However since phosphorylation of the membrane proteins occurred in both the H-7_w and K-1 cells the phenomenon appears to be unrelated to the reduced agglutinability or restriction to concanavalin A receptor mobility observed in the H-7_w cells relative to the K-1 cells. No significant differences in the incorporation of ^{32}P into membrane proteins were observed when either clone was maintained in the presence or absence of 1 mM dibutyryl cyclic AMP.

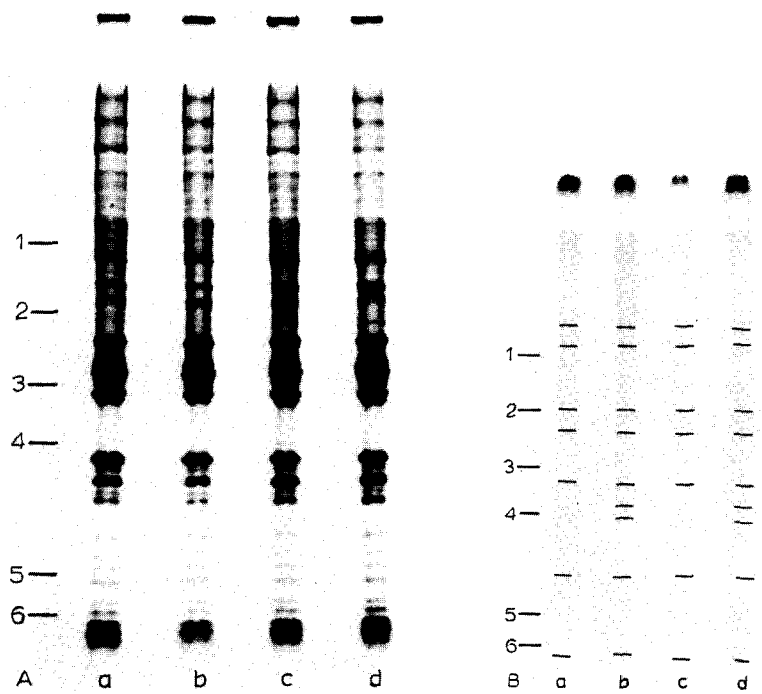

Fig. 9. Phosphorylation of membrane proteins. H-7_w and K-1 cells were maintained for 48 h in media with and without dibutyryl cyclic AMP plus 2.5 μCi/ml [^{32}P]orthophosphate. The cells were removed from the plate as described in Materials and Methods. After the membrane fractions were isolated they were treated with nucleases and the phospholipids extracted as described in Materials and Methods. The membrane proteins were solubilized and equal numbers of counts (~15 000) were applied to the gradient slab gel described in Fig. 3. After separation of the membrane peptides and glycopeptides the gel was stained with Coomassie Brilliant Blue and an autoradiograph produced. (A) Coomassie Brilliant Blue-stained profile. Molecular weight markers (1—6) as in Fig. 3. a, H-7_w; b, H-7_w + dibutyryl cyclic AMP; c, K-1 and d, K-1 + dibutyryl cyclic AMP. It should be noted that nuclease digestion and chloroform/methanol extraction does not change the Coomassie Brilliant Blue staining profile of the membrane peptides (compare with Fig. 3). (B) Autoradiograph of phosphorylated membrane peptides. Molecular weight markers (1—6) as in Fig. 3. a, H-7_w; b, H-7_w + dibutyryl cyclic AMP; c, K-1 and d, K-1 + dibutyryl cyclic AMP. Due to difficulties in photographing the autoradiograph the exposed portions of the film were enhanced prior to making the final print. The final print accurately depicts the relative intensity of the individual bands and, of course, indicates the exact position of the bands.

Leucine labeling of membrane components after medium change

We have previously demonstrated that the addition of dibutyryl cyclic AMP to the growth medium of H-7_w cells results in a restriction to concanavalin A-mediated lectin receptor mobility (Ellison, S., Mulholland, J. and Noonan, K.D., unpublished) and concanavalin A-induced agglutinability [6]. Furthermore, we have demonstrated that this change in surface phenotype occurs within 4 h after the addition of the nucleotide analogue to the growth medium (ref. 6 and Ellison, S., Mulholland, J. and Noonan, K.D., unpublished) (see Fig. 10A). We have also demonstrated in earlier work that the cyclic nucleotide-induced change in the relative agglutinability of the H-7_w cells is dependent on concomitant protein synthesis [6]. This suggested to us that a protein or class of proteins might be added to the CHO membrane during the 4 h time

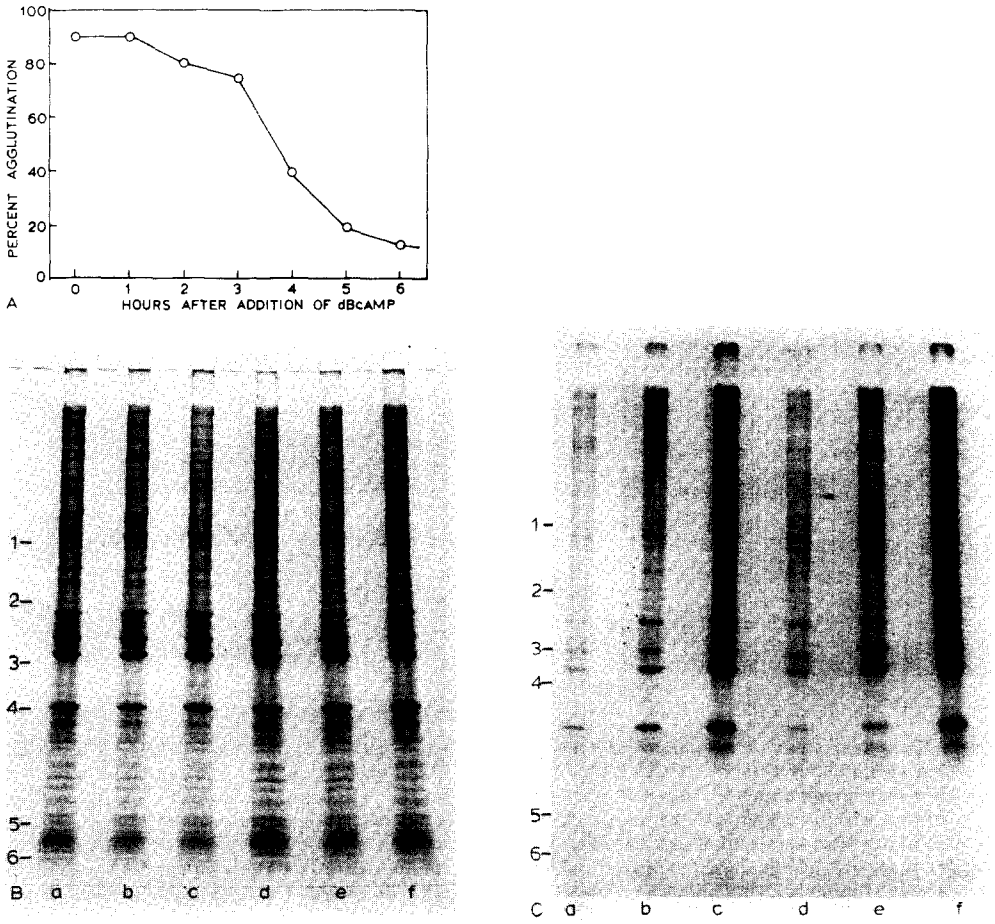

Fig. 10. Incorporation of [^{3}H]leucine during short term incubation with dibutyryl cyclic AMP. H-7$_w$ and K-1 cells were grown for 48 h in the absence of dibutyryl cyclic AMP (dBcAMP). At time 0, medium containing 1 mM dibutyryl cyclic AMP and 3.3 μCi/ml [^{3}H]leucine was added to the cells. The relative agglutinability of the cells was determined every hour after the addition of new media. Membranes were isolated 2, 4, and 6 h after the medium change and both the Coomassie Brilliant Blue staining profile and [^{3}H]leucine incorporation displayed on a gradient slab gel as described in Fig. 3. (A) Relative agglutinability of the cells with 30 μg/ml concanavalin A. (B) Coomassie Brilliant Blue staining profile. 50 μg membrane protein was applied to each slot. Molecular weight markers (1—6) as in Fig. 3. H-7$_w$ + dibutyryl cyclic AMP, 2 (a), 4 (b) and 6 h (c) after medium change; K-1 + dibutyryl cyclic AMP, 2 (d), 4 (e) and 6 h (f) after medium change. (C) Fluorograph of gel displayed in B. Note that equal amounts of protein rather than equal numbers of counts were added to each slot.

period following the addition of dibutyryl cyclic AMP to the growth medium and that such protein(s) might be important in controlling the relative agglutinability of the H-7$_w$ cells with concanavalin A.

In order to determine whether any peptide or class of peptides is specifically synthesized and inserted into the membrane immediately after the addition of the cyclic nucleotide analogue to the growth medium, H-7$_w$ and K-1 cells were maintained for 48 h in McCoy's 5A medium. At time 0, McCoy's 5A plus 1 mM dibutyryl cyclic AMP plus 3.3 μCi/ml [^{3}H]leucine was added to both cell cultures. Membranes were isolated 2, 4 and 6 h after addition of 1 mM

TABLE IV

PHOSPHOLIPID TO PROTEIN RATIOS

Phospholipid extraction and quantitation was performed as described in Materials and Methods.

Cell line	μg phospholipid per mg protein	
	Minus 1 mM dibutyryl cyclic AMP	Plus 1 mM dibutyryl cyclic AMP
H-7_w	192	191
K-1	180	189

dibutyryl cyclic AMP to the growth medium, the peptides separated on a 7.5—12.5% gradient slab gel and then fluorographed. As can be seen in Fig. 10C, essentially all of the membrane components are labeled at 2 h and there is an apparently linear increase in the incorporation of [^{3}H]leucine-labeled components into the membrane over the next 4 h. There is no evidence whatsoever for the preferential insertion of any labeled component or components into the membrane fraction during the 6 h immediately following the addition of dibutyryl cyclic AMP to the growth medium.

Phospholipids and cholesterol

A number of investigators have suggested that changes in membrane fluidity resulting from altered phospholipid or cholesterol composition might account for differences in agglutinability or lectin receptor site mobility [4,5]. We have determined the phospholipid and cholesterol content of H-7_w and K-1 cells maintained in the presence or absence of 1 mM dibutyryl cyclic AMP. As can be seen in Table IV, there are no significant differences in the μg phospholipid per mg protein in the membranous fraction derived from H-7_w or K-1 cells maintained in the presence or absence of dibutyryl cyclic AMP. Similarly we have been unable to detect any substantial differences in the fatty acid composition of the phospholipid fraction extracted from H-7_w cell membranes taken from cells maintained in the presence or absence of 1 mM dibutyryl cyclic AMP (data not shown). The data in Table II indicate that there is essentially no difference in the amount of cholesterol associated with H-7_w cell membranes isolated from cells grown in the presence or absence of dibutyryl cyclic AMP.

Discussion

Intense investigation in many laboratories has been directed towards understanding the molecular basis of concanavalin A-initiated cell agglutination and concanavalin A-directed 'capping' or 'patching' of lectin receptor sites [1]. A number of studies, originating with the work of Burger [2] and Nicolson [3], have suggested that enhanced lectin receptor mobility and concanavalin A-initiated cell agglutination might be controlled by a protease-labile surface component(s). Speculation that agglutination and control of lectin receptor mobility might be controlled by such a surface component was heightened by the work of Hynes and Humphreys [31] and others [32,33] who clearly

demonstrated that many transformed cell lines lacked a high molecular weight glycoprotein (the so-called LETS protein) which, although present on the non-transformed cell surface, was exquisitely sensitive to protease digestion. Further evidence that the LETS protein might play a role in determining the mobility of ligands attached to the cell surface has recently been presented by Albrecht-Buehler and Chen [35].

None of the studies of plasma membrane structure which have so far been presented have, to our knowledge, exclusively addressed the question of what changes in the plasma membrane might be responsible for, or even correlated with, restrictions of concanavalin A-mediated agglutination or receptor site mobility. Furthermore, as far as we know, no attempt has been made to evaluate the membrane composition of genetically identical cell clones which are capable of rapidly modifying concanavalin A-detectable surface phenotypes. In our opinion the CHO subclones which we have used to investigate the relation of membrane composition to changes in surface phenotype are ideal candidates for detecting modifications related to concanavalin A-detected changes in the cell surface. The study of membrane composition presented in this paper indicates that there are no major qualitative or quantitative changes in membrane composition which can be correlated with the concanavalin A-detectable surface phenotypes described in Table I and Fig. 1.

Despite the fact that no major differences in membrane structure have been detected which can be easily correlated with enhanced agglutinability or lectin receptor mobility, a number of observations have been made which we believe are of significance to any future study of the molecular basis of concanavalin A-induced receptor site mobility or concanavalin A-mediated cell agglutination. For example, it is of interest to note that labeling of electrophoretically separated membrane glycopeptides with ^{125}I-labeled concanavalin A failed to reveal any qualitative differences in the concanavalin A binding glycopeptides present in the membrane fraction derived from agglutinable or non-agglutinable cells. This finding suggests that the agglutinable membrane does not express a subclass of concanavalin A receptors which might be responsible for the enhanced agglutinability of the cell type investigated. These data are particularly interesting since Burridge [25] has previously demonstrated the presence of different concanavalin A receptors in homogenates derived from normal and virus-transformed mouse embryo fibroblasts. Our data clearly suggest that these differences detected by Burridge [25] are either unrelated to or, at least, unnecessary for the enhanced concanavalin A agglutinability observed in most transformed cell lines.

Labeling of agglutinable and non-agglutinable cells with glucosamine, $NaIO_4$/NaB^3H_4, galactose oxidase/NaB^3H_4 or ^{125}I all failed to reveal qualitative differences between the membrane fractions isolated from the two cell types. Work carried out with these probes of membrane structure is of particular interest with regard to a glycoprotein of ~260 000 molecular weight which is found on the CHO cell surface and which is undoubtedly analogous to the LETS protein which has been implicated in determining lectin receptor mobility or the relative agglutinability of a cell line [36]. The work reported here clearly demonstrates that in this CHO cell system the presence of the LETS protein on the cell surface does not determine either the relative mobility of the

generalized class of concanavalin A receptors or the relative agglutinability of these CHO cell clones. These data support, and are supported by, previously published data which suggested that there was no necessary correlation between the presence of the LETS protein on the cell surface and either concanavalin A-induced cell-cell agglutination [43] or control of cell growth [44].

The data which we have derived from this CHO cell system with regard to phospholipid, fatty acid and cholesterol composition of the membrane-enriched fraction clearly support the evidence from other laboratories suggesting that changes in the composition of the lipid phase of the plasma membrane is not primarily responsible for controlling the relative agglutinability or lectin receptor site mobility of a cell line [1].

Although it is recognized that the data presented in this manuscript do not resolve the questions underlying the molecular basis of concanavalin A-initiated cell agglutination or concanavalin A-induced receptor site capping, it should be noted that the data derived do speak to two models recently proposed regarding the association of actin cables with the cell membrane and the association of lectin-titrated receptors with actin.

With regard to the association of the actin cables with the cell membrane, Puck [41] has recently suggested, on the basis of data accumulated in his laboratory with the K-1 subclone, that the addition of dibutyryl cyclic AMP to the K-1 cells induces the appearance of a new membrane protein which is capable of associating with the intracellular actin cables. According to Puck's hypothesis this 'new' membrane protein would provide a site for stress fiber attachment to the cell membrane. Furthermore Puck suggests that this attachment of the actin cables to the membrane is responsible for the dibutyryl cyclic AMP-induced change in the CHO cells' morphology [30]. Clearly our data strongly suggest that no new membrane proteins are associated with the CHO cell membranes when the cells are maintained in medium supplemented with dibutyryl cyclic AMP. Thus our data force us to conclude that either a more subtle change in a pre-existing membrane protein is responsible for the association of the actin cables with the cell membrane or that the hypothetical actin binding protein proposed by Puck [41] does not exist.

Singer and his collaborators [42] have recently suggested that in order for lectin-titrated receptors to cap, the receptors must associate with a hypothetical integral membrane protein X which, in turn, is associated with the actin filaments of the cell. If this hypothesis is applicable to the CHO cells studied then one might expect that the cells capable of capping the majority of their concanavalin A receptors (i.e. the H-7_w and K-1 cells as well as K-1 cells maintained in medium supplmented with 1 mM dibutyryl cyclic AMP) would display a membrane protein which would be absent from CHO cells which are incapable of capping the concanavalin A receptors (i.e. the H-7_w cells maintained in medium supplemented with 1 mM dibutyryl cyclic AMP). Clearly the data presented in this manuscript do not immediately suggest the existence of such a protein. These data could be taken to suggest that either the hypothetical membrane component X is present in very low molar quantities or does not exist. Our data allows us to develope alternate approaches for seeking the hypothetical X protein and thus serves us very well in our on-going search for

the molecular basis of concanavalin A-initiated receptor site capping.

Although our data clearly show that there are no major dibutyryl cyclic AMP-induced qualitative or quantitative changes in the membrane composition of the two CHO subclones investigated, we cannot rule our the possibility that less 'dramatic' changes in membrane composition exist. What our data indicate is that any changes in membrane composition which might occur when these cell clones are maintained in dibutyryl cyclic AMP must be relatively subtle changes not readily detectable by standard procedures of membrane isolation and characterization. Furthermore, our data suggest that the differences which have previously been described between normal and transformed cell membranes (e.g. refs. 31 and 37) are probably not responsible for the lectin-detected surface phenotypes of those cell lines.

Acknowledgements

This work was supported by U.S.P.H.S. Grant GM-0994. I would like to acknowledge the expert technical assistance of Ms. Siobhan Ellison as well as the cooperation of Dr. Carl M. Feldherr in obtaining the electron micrographs. I would also like to thank Dr. A.W. Hsie (Oak Ridge National Laboratories) for first providing us with the CHO cells from which our subclones have been derived and Dr. James Starling for providing the concanavalin A overlay autoradiograph.

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