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cAMP-Dependent Protein Kinase Modulates Expression and Subcellular Localization of Dolichos Biflorus Agglutinin Binding Sites in Renal Epithelial Cells

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Abstract Previous studies demonstrated that, upon attaining confluence, a clone of the renal epithelial cell, LLC-PK_1, expressed progressively binding sites for the lectin Dolichos biflorus agglutinin (DBA) at the apical cell surface. Activation of cAMP-dependent protein kinase enhanced surface expression dramatically. The goal of this study was to define the process leading to surface expression of DBA binding sites and to investigate further the role of cAMP-dependent protein kinase in modulating surface expression. Both subconfluent and confluent cells exhibited intracellular DBA binding sites (50–70% of total cellular binding sites) in a perinuclear vesicular compartment which was disrupted by Brefeldin A treatment. Both total cellular content and the proportion of DBA binding sites at the cell surface increased modestly after confluence was attained. A 48 h treatment of cells with 1-methyl-3-isobutyl xanthine, a phosphodiesterase inhibitor, dramatically increased the level of cellular DBA binding sites as well as the proportion of DBA binding sites at the cell surface. Analysis of two mutants of this cell line suggests that the effect of 1-methyl-3-isobutyl xanthine requires cAMP-dependent protein kinase activity but is *not* due to cAMP-dependent protein kinase-mediated activation of gene transcription.

Key words: Golgi compartment, BBM, glycosylation, LLC-PK_1 cell, apical protein sorting

Several studies have demonstrated preferential binding of lectins to a specific cell type and/or membrane domain, including renal epithelial cells [see, e.g., Schuster et al., 1986]. Confluent populations of the pig renal epithelial cell, LLC-PK_1, expressed a typical apical, or brush border membrane (BBM) [Amsler et al., 1991; Mullin et al., 1980; Pfaller et al., 1990]. Binding sites for the lectin, Dolichos biflorus agglutinin (DBA), were localized preferentially to the BBM in postconfluent populations of a clone (Cl4) of this cell line. BBM DBA binding capacity increased dramatically upon activation of the cAMP-dependent protein kinase (PKA).

Appearance of DBA binding sites at the BBM could be produced by one or both of two distinct mechanisms. First, the total content of DBA binding sites in the cell could increase progressively and the proportion of the total sites expressed at the BBM could remain constant. Second, the total content of binding sites could remain constant but the proportion of sites expressed at the BBM could increase progressively.

The aims of this study were 1) to define the subcellular localization of DBA binding sites in Cl4 cells and 2) to begin to elucidate the process leading to enhanced BBM DBA binding capacity under control conditions and upon activation of PKA.

METHODS

Cell Culture

The clone of the LLC-PK_1 cell line used in this study (Cl4) was a gift from Dr. J.S. Cook (Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN). The original line was a gift from Dr. R.N. Hull (Eli Lilly Laboratory, Indianapolis, IN). The mutants of the LLC-PK_1 cell line were a gift from Dr. B.A. Hemmings (Friedrich-Miescher Institute, Basel, Switzerland). Cells were maintained in stock culture at a subconfluent density in Complete Medium (alpha modification of Eagle's Minimal Essential Medium supplemented with 10% fetal bovine serum) at 37°C in a humidified 5% CO_2 atmosphere.

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For experiments, cells were detached from stock cultures by treatment with trypsin-EDTA and seeded at a density of approximately 5×10^4 cells per 35 mm cell culture dish in Complete Medium. Cultures were incubated as above. After 2 days (populations were still subconfluent), medium was aspirated and replaced with Complete Medium without or with 200 μM 1-methyl-3-isobutyl xanthine (MIX; a potent inhibitor of phosphodiesterase). Confluence was routinely attained 2–3 days after this medium change. Medium was replenished every 2 to 3 days.

Lectin Binding

Medium was aspirated and populations were rinsed twice with ice cold Hanks' Balanced Salt Solution buffered with 10 mM Hepes-Tris, pH 7.2 (HBSS). One milliliter ice-cold HBSS without or with 100 mM competitive sugar (N-acetyl-D-galactosamine) was added to each culture followed by 20 μl HBSS containing fluorescein-DBA (final concentration: 5–25 μg/ml; Vector Laboratories, Burlingame, CA). Populations were incubated at 4°C with rocking for 30 min. Binding was terminated by aspirating solution and rinsing four times with ice cold isotonic salt solution (150 mM NaCl, 2 mM Tris-HCl, pH 7.2). After drying, protein and bound fluorescein-DBA were solubilized by addition of 2% SDS Solution (2% SDS, 10 mM Tris, 1 mM EDTA, pH 9.2). Bound DBA was quantitated as arbitrary fluorescence units (excitation wavelength, 495 nm; emission wavelength, 525 nm). Protein was quantitated by a fluorescence assay [Avruch and Wallach, 1971].

Specific lectin binding is calculated as the difference between binding in the absence and presence of competitive sugar. Inclusion of non-competitive sugar at 200 mM did not affect binding. Preliminary experiments demonstrated that 1) measurement of protein using the fluorescence assay was not affected by the presence of fluorescein-lectin, 2) 100 mM sugar was sufficient to produce near-maximal inhibition of DBA binding, and 3) 30 min incubation was sufficient to attain maximal specific binding.

Data are expressed as mean ± standard deviation of triplicate, independent samples. Where error bars are not visible, they are smaller than the symbols. When calculating the proportion of DBA binding sites expressed at the BBM, data are expressed as the ratio of surface to total cellular DBA binding. Each of these values (surface and total) represents a mean ± standard deviation of triplicate independent samples. Because different amounts of lectin were used in different experiments and the level of binding is expressed in arbitrary fluorescence units, which are entirely dependent on the settings of the fluorometer, quantitative comparison between experiments is not possible. Regardless of the absolute numbers, however, the qualitative changes observed were reproducible from day to day and experiment to experiment.

To quantitate total cellular DBA binding sites, populations were pretreated with 95% ethanol for at least 30 min at room temperature. Following this pretreatment, populations were processed as described above. Ethanol treatment permeabilized cells and permitted access to intracellular components, as evidenced by DBA labeling of intracellular vesicles (see Results) and binding of antibodies to villin and myosin, two intracellular cytoskeletal proteins (unpublished observation) in the ethanol-treated, but not in the untreated cell populations.

TABLE I. DBA Binding Site Content and Distribution of Post-Confluent Populations of Cl4 Cells Maintained in the Absence and Presence of 200 μM MIX*

Sample	DBA binding capacity: arbitrary fluorescence units / mg cell protein	Ratio (percent)
Control		
Intact	268 ± 26	38
Ethanol-permeabilized	699 ± 27	
+200 μM MIX		
Intact	1,133 ± 14	96
Ethanol-permeabilized	1,177 ± 28	

*Post-confluent Cl4 cell populations maintained in the absence and presence of 200 μM MIX for 7 days. Ethanol treatment of cell populations was performed as described in Methods. Binding of fluorescein-DBA was performed as described in Methods using triplicate, independent cultures. Data are expressed as the mean ± standard deviation.

For fluorescence microscopy, populations were treated as described above. After rinsing to remove free DBA, 2 ml ice-cold HBSS was added and populations were kept on ice until viewing. To ensure our ability to compare visually the extent of DBA binding recorded in different photographs, the same exposure time, camera setting, and development time were used for all photographs taken during a single experiment.

RESULTS

Cl4 Cells Contain Intracellular DBA Binding Sites

The lectin, DBA, bound to the surface of postconfluent Cl4 cells (Table I). Parallel populations permeabilized with ethanol prior to incubation with DBA exhibited a significantly greater level of binding (approximately three-fold greater), indicating that control cell populations possess a significant subpopulation of total DBA binding sites in a compartment which is not accessible to external DBA.

DBA binding to the surface of Cl4 cell populations maintained for many days in the presence of 200 μM 1-methyl-3-isobutyl xanthine (MIX) was much greater than binding to the surface of control cell populations (Table I). MIX-treated cell populations permeabilized with ethanol prior to lectin addition bound an approximately equivalent amount of DBA to unpermeabilized populations, indicating that in MIX-treated populations a vast majority of DBA binding sites were accessible to externally applied DBA. Total DBA binding capacity of MIX-treated cell populations was also increased compared to control cell populations (approximately 60% greater).

Visualization of Intracellular DBA Binding Sites

To localize the inaccessible DBA binding sites, postconfluent Cl4 cell populations were permeabilized by treatment with ethanol and then reacted with fluorescein-DBA (Fig. 1b). Parallel cell populations were reacted with fluorescein-DBA without ethanol permeabilization (Fig. 1a).

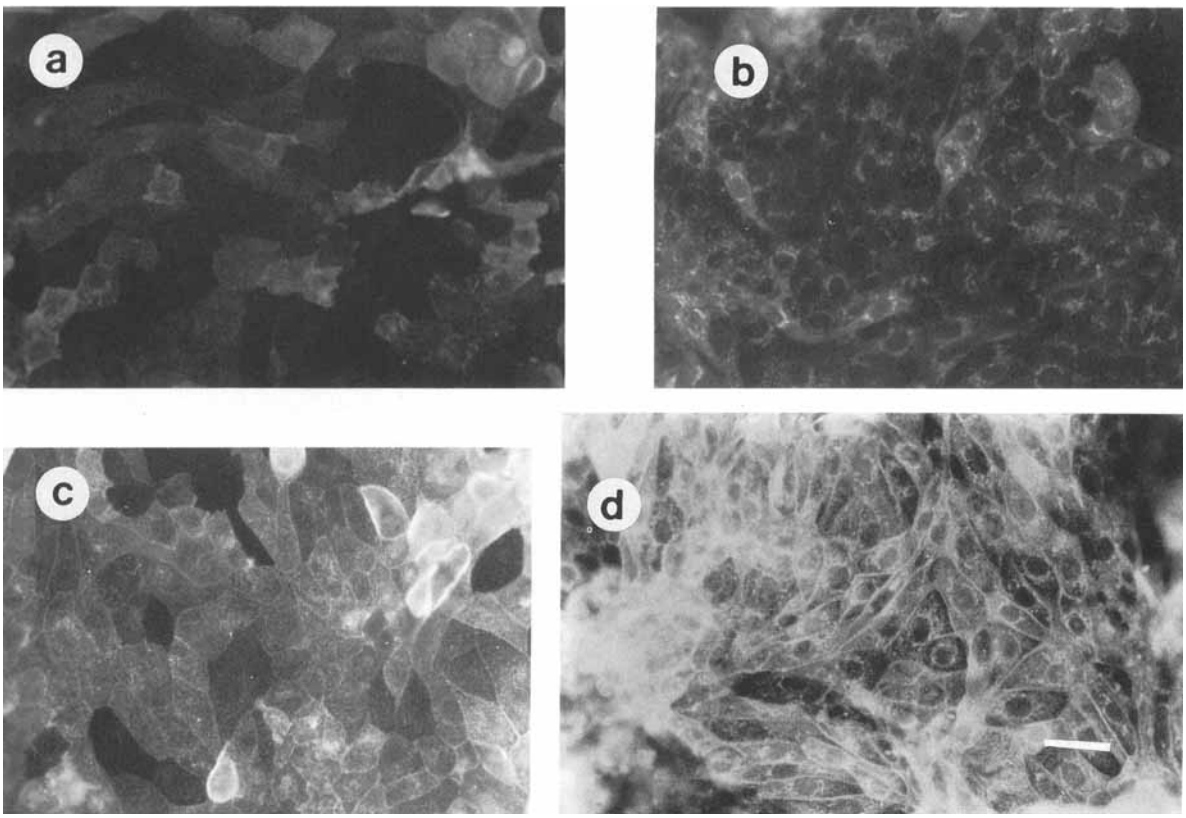

Fig. 1. Visualization of surface and intracellular DBA binding sites in postconfluent Cl4 cell populations maintained in the absence (**a** and **b**) or presence (**c** and **d**) of 200 μM 1-methyl-3-isobutyl xanthine Prior to DBA binding, some populations were permeabilized with 95% ethanol (b and d), while in other populations DBA binding was visualized in intact cells (a and c) Binding of fluorescein-DBA was performed as described in Methods Fluorescein-DBA was visualized using a Zeiss IM-35 microscope equipped for epifluorescence Bar in the lower right-hand corner of (d) represents 20 μm

Inclusion of 100 mM of the competitive sugar N-acetyl-D-galactosamine dramatically reduced the level of DBA binding to all labeled structures (data not shown).

The binding of DBA to intact cells was highly heterogeneous (Fig. 1a). Some cells bound virtually no DBA and other cells bound varying amounts of DBA. In labeled cells, DBA binding sites were distributed over the entire cell surface. Some labeled cells exhibited a punctate distribution of DBA, suggestive of labeling of microvilli.

The differential labeling pattern for intact cells is contrasted with the labeling of most, if not all, cells pretreated with ethanol (Fig. 1b). DBA binding sites were detected in vesicular structures in a perinuclear location. Nuclei were clearly visible as darker ovals, indicating that the nuclear membrane possesses relatively few DBA binding sites. In addition to labeling of the vesicular structures, many cells displayed a weak, diffuse labeling with DBA, whereas some cells exhibited stronger homogeneous staining. Since binding of DBA could not be detected to the surface of many cells (see Fig. 1a), the weak, diffuse labeling with DBA probably reflects binding to intracellular binding sites distributed throughout the cytoplasm of most cells. The stronger homogeneous staining probably represents labeling of cell surface binding sites.

We also visualized DBA binding to intact and ethanol-permeabilized postconfluent Cl4 cells maintained in the presence of 200 μM MIX for 7 days. Intact, MIX-treated Cl4 cells also exhibited differential labeling with DBA (Fig. 1c). Although most cells were labeled to some extent, no binding could be detected to a few cells. Many cells exhibited a punctate distribution of surface label. The labeling intensity of the cell surface was noticably stronger in MIX-treated as compared to control cell populations (compare Fig. 1a and c), consistent with the quantitation in Table I.

Ethanol-permeabilized, MIX-treated Cl4 cells were strongly labeled with DBA (Fig. 1d). Virtually all cells exhibited the homogeneous staining which likely represents labeling of the cell surface, although a portion of this labeling may also represent binding to diffuse intracellular sites. Vesicular structures in the perinuclear region were strongly labeled with DBA. The labeling of the vesicular structures appeared markedly stronger in ethanol-permeabilized MIX-treated cell populations as compared to ethanol-permeabilized control cell populations (compare Fig. 1b and d).

Effect of Brefeldin A Treatment on the Localization of Intracellular DBA Binding Sites

Brefeldin A (BFA) blocks recycling of membranes between the ER and the Golgi, causing accumulation of Golgi proteins in ER structures [Lippincott-Schwartz et al., 1989, 1991], and has been used to confirm localization of cellular components to the Golgi network [Ridgeway et al., 1992]. To determine if the vesicular structures labeled with DBA in ethanol-permeabilized cells are components of the Golgi network, we examined the labeling by DBA of cells treated without and with 5 μg/ml BFA for 3 h and then permeabilized with ethanol (Fig. 2).

DBA binding sites in postconfluent populations of Cl4 cells were localized primarily to perinuclear vesicular structures (Fig. 2a), as

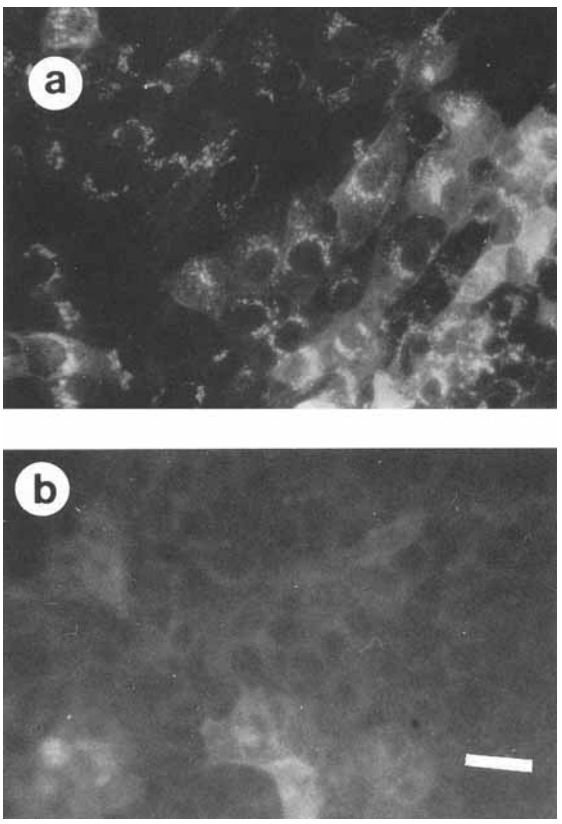

Fig. 2. Effect of Brefeldin A treatment on the localization of DBA binding sites contained in the vesicular structures in Cl4 cells. Cell populations were maintained in culture until postconfluent and then treated for 3 h without (**a**) or with (**b**) 5 μg/ml Brefeldin A. Populations were permeabilized with 95% ethanol and localization of DBA binding sites was determined using fluorescein-DBA as described in Methods. Bar in the lower right-hand corner of (b) represents 10 μm.

described above. In cells treated with BFA these brightly staining vesicular structures were almost completely dispersed and the cells displayed a weak, diffuse staining throughout the cytoplasm (Fig. 2b). This supports the hypothesis that the vesicular structures containing DBA binding sites are a part of the Golgi compartment. BFA treatment produced a similar dispersal of vesicular binding sites in both postconfluent control and MIX-treated cell populations (data not shown).

DBA Binding Sites Are Redistributed From the Intracellular Compartment to the Cell Surface

The above results indicated that postconfluent Cl4 cells contain a substantial number of DBA binding sites in a compartment which is not accessible to added DBA in intact cells but is accessible in ethanol-permeabilized cells. Further, the results suggest that MIX treatment produced a redistribution of DBA binding sites from this inaccessible compartment to an accessible compartment. To investigate this in more detail, the number of DBA binding sites was determined in intact and ethanol-permeabilized cells as a function of time in which the cell populations were maintained in the absence or presence of 200 μM MIX (Fig. 3). Figure 3a presents the quantitation of DBA binding sites in intact and ethanol-permeabilized control Cl4 cell populations. Figure 3b quantifies the DBA binding sites in intact and ethanol-permeabilized MIX-treated Cl4 cell populations. Figure 3c presents the ratio of DBA binding sites in intact and ethanol-permeabilized cells in both control and MIX-treated Cl4 cell populations.

On day 0 of this experiment (subconfluent), cells displayed a low level of DBA binding in both intact and permeabilized cell populations (Fig. 3a). The ratio of binding sites in intact and ethanol-permeabilized cell populations was about 30% (Fig. 3c). By day 2 (just-confluent), control cell populations exhibited an increase in the number of DBA binding sites in both intact (about 2-fold) and permeabilized (about 50%) cells (Fig. 3a). The ratio increased to 44% (Fig. 3c). Throughout the remainder of this experiment, the number of binding sites accessible in intact cells and in permeabilized cells increased modestly. There was a progressive increase in the ratio of DBA binding sites which were labeled in intact and ethanol-permeabilized cells to about 60% by the end of the experiment.

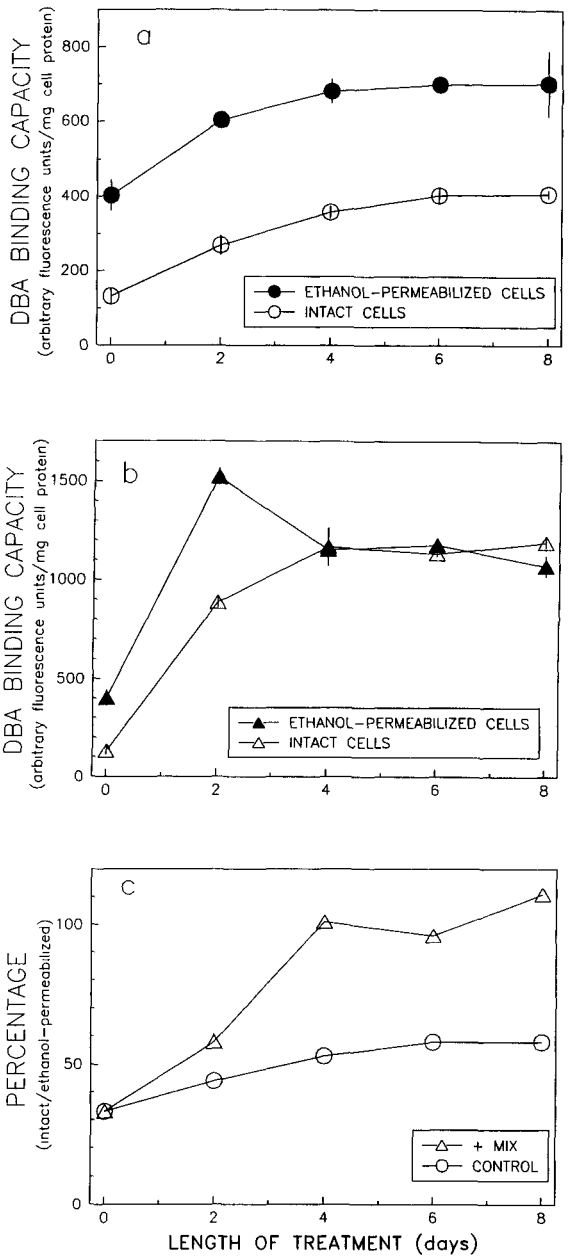

Fig. 3. Quantitation of DBA binding sites in Cl4 cell populations maintained in the absence (**a**) and presence (**b**) of 200 μM 1-methyl-3-isobutyl xanthine Cell populations were seeded at a subconfluent density in Complete Medium After 2 days populations were refed with the desired medium Medium was replenished every 2 days DBA binding capacity was measured as described in Methods either directly or following ethanol treatment Data in a and b are presented as mean ± standard deviation of triplicate independent samples Data in c are the ratios of the means of the binding capacities presented in a and b measured on parallel samples

Cell populations maintained in the presence of 200 μM MIX possessed more DBA binding sites in both compartments (Fig. 3b) compared to control cell populations (Fig. 3a) throughout

the experiment. The level of DBA binding to ethanol-permeabilized cells reached a maximum after 2 days in MIX (Fig. 3b) and then remained relatively constant thereafter. In contrast, the level of DBA binding to intact cells increased progressively up to 4 days in MIX. The ratio of DBA binding sites in intact and ethanol-permeabilized cells increased progressively over the first 4 days in MIX and remained constant thereafter (Fig. 3c).

MIX-Induced Changes in Capacity and Distribution of DBA Binding Sites Is More Pronounced in Postconfluent Compared to Subconfluent Cell Populations

The results presented in Figure 3 suggested that the MIX effect on the total content and distribution of DBA binding sites might be more pronounced in confluent or postconfluent cell populations than in subconfluent cell populations. To examine this possibility directly, we compared the effect of a 48 h MIX treatment on postconfluent and subconfluent populations of Cl4 cells (Table II). Compared to control, postconfluent Cl4 cell populations, MIX treatment of postconfluent cell populations for 48 h produced a substantial increase in DBA binding capacity of intact cells and a smaller increase in binding capacity in ethanol-permeabilized cells. The percentage of the binding sites expressed at the cell surface in the two populations increased from 37% in control cell populations to 95% in MIX-treated cell populations.

In contrast, MIX treatment of subconfluent cell populations (approximately 40% of confluence at the time of measurement of DBA binding capacity) produced little change in DBA binding capacity of either compartment compared to parallel control cell populations. Binding capacity in ethanol-permeabilized cell populations remained relatively unchanged by MIX treatment and produced a doubling of DBA binding capacity in intact cell populations. The ratio of DBA binding sites expressed in the two compartments increased from 15 to 33%.

The Role of PKA in MIX-Induced Changes in DBA Binding Site Capacity and Localization

Addition of MIX alone induces changes in cellular cAMP content of LLC-PK_1 cells [Amsler and Cook, 1982]. Changes in cellular behavior produced by elevation of cAMP content are generally attributed to cAMP activation of PKA; however, this is rarely tested explicitly. While many PKA-mediated changes in cell behavior are due to activation of the transcription of specific genes [Gonzalez and Montminy, 1989], direct modulation of enzyme and transport activities by PKA-mediated phosphorylation is also well documented [see, e.g., Cheng et al., 1991].

TABLE II. Effect of MIX on DBA Binding Site Localization and Content in Post-Confluent and Subconfluent Cl4 Cell Populations*

Sample	DBA binding capacity (arbitrary fluorescence units / mg cell protein)	Ratio (percent)
Post-confluent		
Control		
Intact	588 ± 28	37
Ethanol-permeabilized	1,610 ± 16	
+200 μM MIX		
Intact	2,466 ± 40	95
Ethanol-permeabilized	2,588 ± 105	
Subconfluent		
Control		
Intact	106 ± 4	15
Ethanol-permeabilized	709 ± 90	
+200 μM MIX		
Intact	234 ± 14	33
Ethanol-permeabilized	717 ± 44	

*Subconfluent and post-confluent populations of Cl4 cells were maintained in the absence and presence of 200 μM MIX for 48 h. Intact and ethanol-permeabilized cell populations were prepared and DBA binding was performed and quantitated as described in Methods. Data are expressed as mean ± standard deviation of triplicate, independent samples.

To determine whether or not PKA is required for the MIX-induced effects on DBA binding site capacity and distribution, and to provide insights into whether the requirement reflects an activation of gene transcription or modulation of an enzymatic activity, we compared the effect of 48 h MIX treatment on DBA binding capacity in the intact and ethanol-permeabilized populations of Cl4 cells and populations of two mutants of the LLC-PK_1 cell. One mutant, FIB4, is deficient in PKA activity [Botterell et al., 1987]. The second mutant, FIB5, exhibits normal PKA activity but is unable to activate transcription of a transfected gene under control of the cAMP-Regulatory Element (CRE) promoter [Catanzariti, 1992].

Control Cl4 cell populations displayed DBA binding in both intact and ethanol-permeabilized cell populations, as described above. The ratio of binding in the two populations was about 20% (Table III). In this experiment, exposure of cell populations to MIX for 48 h produced a relatively small increase in DBA binding capacity in ethanol-permeabilized cells (39%) but a dramatic increase in DBA binding to intact cells. Thus, MIX treatment increased the ratio of binding in the two populations to about 77%.

Populations of FIB4 cells (PKA-deficient) maintained in the absence of MIX expressed DBA binding sites both in intact and ethanol-permeabilized cell populations (Table III). The ratio of DBA binding to these populations was about 43%. Inclusion of MIX in the culture medium for 48 h did not increase DBA binding capacity in either cell population. This supported the hypothesis that PKA activity was required for MIX to modulate DBA binding capacity in both compartments.

Populations of FIB5 cells (PKA-mediated transcription-deficient) maintained in the absence of MIX expressed substantially more DBA binding sites in ethanol-permeabilized as compared to intact cell populations, as expected (Table III). The ratio of binding capacity in these two populations was about 39%. Exposure of FIB5 cell populations to MIX for 48 h produced a dramatic increase in DBA binding capacity in both intact and ethanol-permeabilized cell popula-

TABLE III. Effect of MIX on DBA Binding Site Content and Localization in High Density Populations of Cl4 Cells, FIB4 Cells, and FIB5 Cells*

Sample	DBA binding capacity arbitrary fluorescence units mg cell protein	Ratio (percent)
Cl4		
Control		
Intact	227 ± 19	20
Ethanol-permeabilized	1,122 ± 71	
+200 μM MIX		
Intact	1,207 ± 98	77
Ethanol-permeabilized	1,563 ± 117	
FIB4		
Control		
Intact	277 ± 4	43
Ethanol-permeabilized	646 ± 108	
+200 μM MIX		
Intact	123 ± 16	29
Ethanol-permeabilized	428 ± 44	
FIB5		
Control		
Intact	330 ± 28	39
Ethanol-permeabilized	839 ± 27	
+200 μM MIX		
Intact	1,978 ± 297	85
Ethanol-permeabilized	2,327 ± 44	

*Populations of cells were maintained in Complete Medium until several days post-confluent Medium was then aspirated and replaced with Complete Medium ± 200 μM MIX for 48 h Cell populations were prepared for binding and DBA binding was quantitated as described in Methods Data are expressed as mean ± standard deviation of triplicate independent samples

tions. The ratio of DBA binding sites expressed by the two cell populations was about 85%. This result suggests that the MIX-induced changes in DBA binding site capacity and distribution do not require PKA-mediated activation of gene transcription.

DISCUSSION

In this study we have provided evidence for a PKA-mediated modulation of the total cellular content and subcellular distribution of binding sites for the lectin Dolichos biflorus agglutinin in a renal epithelial cell line. These two effects may be distinct processes which are both modulated by PKA or may reflect two manifestations of a single process.

DBA binds to terminal alpha-linked N-acetyl-D-galactosamine residues [Piller et al., 1990]. An increase in total DBA-binding capacity must be due to an increased cellular content of glycoconjugates containing this terminal sugar. The vast majority of DBA-binding sites are glycoproteins and DBA binds to a similar set of cellular glycoproteins under all conditions examined (manuscript in press), indicating that the PKA-mediated increase in cellular DBA-binding capacity does not reflect PKA-induced expression of unique DBA-binding glycoproteins. Rather, PKA activation must increase the cellular content of DBA-binding glycoproteins which are present under normal conditions.

An increased cellular content of DBA-binding glycoproteins could be produced either by an increase in the cellular content of proteins which receive this (these) DBA-binding oligosaccharide(s) or by an increase in the rate of synthesis and/or transfer of this(these) DBA-binding oligosaccharide(s) to proteins without a change in cellular content of the acceptor proteins. The ability of FIB5 mutant cells, which are deficient in PKA-dependent activation of gene transcription but contain normal PKA activity, to increase DBA-binding capacity upon MIX treatment strongly suggests that the MIX-induced increase in DBA-binding capacity does not require a PKA-mediated activation of gene transcription. Thus, it seems likely that the MIX-induced increase in cellular DBA-binding site content reflects a PKA-dependent change in the synthesis, processing, and/or transfer of DBA-binding oligosaccharides to existing proteins. We cannot rule out the possibility that the PKA-dependent increase in DBA binding site content reflects a PKA-mediated enhancement of translation of the protein component of these glycoproteins. Since MIX treatment does not produce a general increase in protein synthetic rate (unpublished observation), however, PKA would have to stimulate preferentially the translation of specific mRNAs.

The MIX-induced increase is dependent on protein synthesis (manuscript submitted). Since data presented here suggest that the response does not involve PKA-mediated gene transcription, this protein synthesis-dependence probably reflects the existence of a rapidly turning-over protein(s) which is essential for synthesis of the DBA-binding glycoproteins.

Under control conditions, Cl4 cells expressed a significant proportion of DBA binding sites in a compartment which was not accessible to externally added DBA. Most of these binding sites were localized to a perinuclear vesicular compartment which was disrupted by BFA. This suggests that these DBA-binding sites are localized within the cell in the Golgi compartment [Ridgeway et al., 1992]. The Golgi compartment is involved in posttranslational processing of glycoproteins, including processing of the core high-mannose oligosaccharide chains which are attached in the endoplasmic reticulum into mature complex oligosaccharide chains. Therefore, the PKA-mediated increase in total DBA binding capacity may reflect modulation of an oligosaccharide processing/transfer step occurring within the Golgi compartment.

MIX treatment of near-confluent cell populations produced a dramatic increase in total DBA binding capacity which was not increased further despite continued presence of MIX (Fig. 3). As shown in Table II, acute (48 h) MIX treatment of very subconfluent cell populations did not produce an increase in the total DBA binding capacity whereas treatment of postconfluent cell populations did exhibit an increase. Thus, the PKA-mediated increase in DBA binding capacity only occurs when cell populations are at or very near to confluence. This induction appears to be maximal after 48 h since chronic MIX treatment does not increase further the magnitude of this effect. The results suggest that a necessary component must first be expressed for the PKA-mediated effect to be evident and that this component is only expressed at confluence. The nature of this component is unknown but may be a part of the cellular oligosaccharide processing or transfer machinery present only in polarized cells (see below).

Distribution of DBA binding sites was also modulated by PKA activity. Under control conditions, most of the binding sites were contained in this putative Golgi compartment. Activation of PKA induced a redistribution of these binding sites such that a larger proportion were expressed at the cell surface. Although the quantitative data from some experiments suggested a complete redistribution of DBA binding sites to the cell surface, the fluorescence micrographs demonstrate that most MIX-treated cells still express DBA binding sites in these vesicular structures (see, e.g., Fig. 1d). Quantitation of DBA binding to cell populations which were ethanol-permeabilized directly versus populations which were fixed with glutaraldehyde prior to ethanol permeabilization suggested that ethanol permeabilization of unfixed cell populations extracted a variable amount (never more than 20%) of the DBA binding sites (unpublished observation). Thus, our measurements of binding site capacity should be regarded as semiquantitative. Assuming the maximum inaccuracy in quantitation of total cellular DBA binding capacity introduced by this procedure, the data still indicate that the proportions of binding sites in the putative Golgi compartment and at the cell surface were markedly altered by MIX treatment. This demonstrates that the redistribution of DBA binding sites documented in this report is a real physiological event rather than an artefact of our measurement procedure.

One potential explanation for this MIX-induced alteration in binding site localization is that PKA induced a retargetting of synthesized DBA binding sites from the putative Golgi compartment to the cell surface. Protein phosphorylation can directly regulate protein targetting [Casanova et al., 1990; Herman et al., 1991] and fusion of intracellular vesicles with the plasma membrane [Brown, 1989].

Alternatively, PKA could indirectly modulate the subcellular localization of glycoproteins by controlling the nature of the attached oligosaccharide. Although many studies have suggested that glycosylation does not affect protein targetting [see, e.g., Green et al., 1981], some studies have provided evidence suggesting that protein glycosylation can in some circumstances affect protein subcellular localization and/or stability [Hoe and Hunt, 1992].

Two recent findings in a cell line, HT-29, which differentiates into an enterocyte-like cell in culture, may bear on the results reported here and may suggest a scenario in which both subcellular localization and glycosylation could be distinct manifestations of the same process. In one study, a change in oligosaccharide processing was associated with differentiation of the cells [Ogden-Denis et al., 1989]. Undifferentiated HT-29 cells did not process oligosaccharide chains to the mature form, whereas, processing to mature oligosaccharide chains was an early marker of expression of the differentiated phenotype.

In a second study, the subcellular localization of an apical membrane protein was modulated during differentiation [Morris et al., 1992]. In rapidly growing, undifferentiated cells, the protein was confined to an intracellular compartment. Appearance of the protein at the apical cell surface occurred coincident with, and was dependent on, appearance of a BFA-sensitive intracellular compartment.

Perhaps construction of an apical membrane domain by some cultured epithelial cells requires synthesis or organization of the intracellular sorting machinery in a compartment which exhibits unique oligosaccharide processing. If so, then an increased cellular content of DBA binding sites and redistribution of DBA binding sites to the apical membrane would be distinct manifestations of the single process by which synthesized apical membrane proteins are processed and sorted to the apical membrane. More work is required to test this hypothesis.

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