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CELL KINETICS OF DIFFERENTIATION OF Na^+-DEPENDENT HEXOSE TRANSPORT IN A CULTURED RENAL EPITHELIAL CELL LINE

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Fully differentiated cells of the renal proximal tubule have the capability of taking up hexoses across their apical borders by transport coupled to the Na^+-electrochemical gradient. This property is also found in postconfluent cultures of the cloned cell line LLC-PK_1, derived from juvenile pig kidney by R. N. Hull (1). But when the cells are maintained as subconfluent, vigorously growing cultures, this differentiated activity is not detectable. The changeover from an undifferentiated, active growth phase to a nongrowing differentiated state in culture presents the opportunity to study the cell kinetics involved in the development of the transport system.

The transport properties of this cell line as a function of growth state in culture have been previously described (reviewed in ref 2). Our standard assay for Na^+-dependent transport is the 60-min uptake of alpha methylglucoside (AMG) from a concentration of 0.1 mM in Hanks balanced salt solution (HBSS). This glucose analog is neither metabolized nor a substrate for the facilitated diffusion transporter at the basolateral surfaces of these polarized cells. Thus, depending on the transport capacity at the apical surface, the analog may be concentrated to high levels within the cells. We maintain stocks in continuous growth during which the Na^+-dependent hexose uptake is not expressed. When such stocks are plated onto collagen-coated filters and cultured, the population on each filter comes into confluence in about two days. A day or so later Na^+-dependent uptake becomes discernible (Fig. 1) and increases over the next two or three weeks, reaching very high levels. Differentiation of this function may be accelerated with cAMP phosphodiesterase inhibitors like dibutyryl cAMP, theophylline, or methyl isobutylxanthine (MIX) and conversely can be inhibited with the tumor promoter tetradecanoyl phorbol acetate (TPA).

Most studies that have been done on this system have been on populations or vesicles derived from populations. It has become apparent that the increasing transport capacity of these populations with time is a direct consequence of an increase in the number of transporters, assayed as the number of Na^+-dependent phlorizin-binding sites (4,5). This is still a population measurement. Our concern has been to analyze the developmental process at the cellular level. The possibilities were that: (1) at the onset of differentiation all cells in

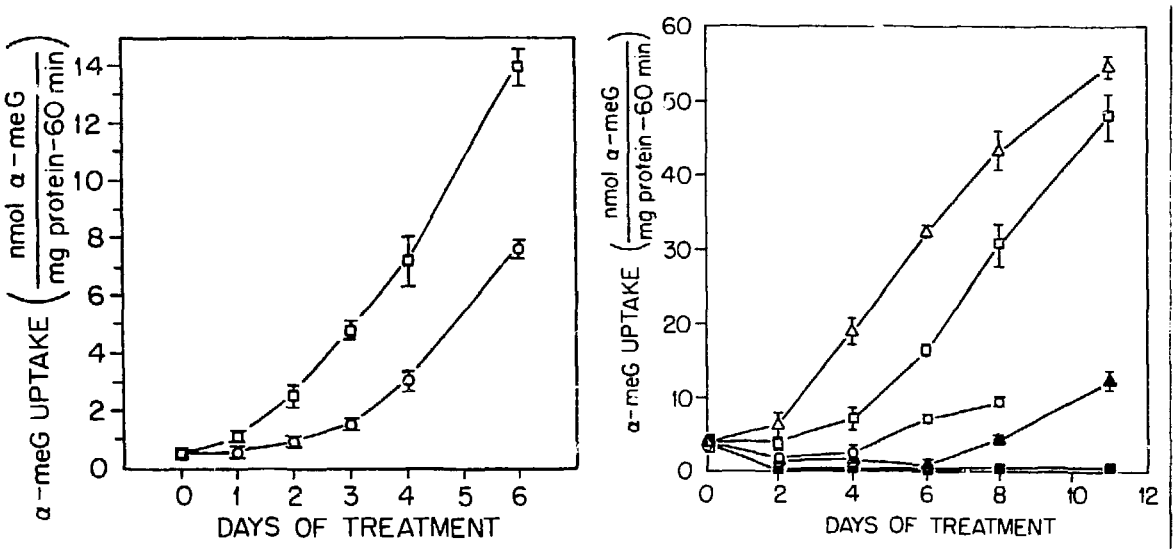

Fig. 1. (A) Early onset of AMG (α-meG) uptake in controls (circles) and cells cultured in 0.25 mM dibutyryl cAMP + 1 mM theophylline (squares). (B) Effects of 1 mM MIX (open triangles), 1 mM theophylline (open squares), 1 mM MIX + 100 nM TPA (closed triangles), and 1 mM theophylline + 100 nM TPA. Controls: open circles. [From ref. 3 with permission.]

the population become synchronously recruited into the differentiated state and, in parallel, develop over a period of weeks their full complement of transporters; (2) at confluence the undifferentiated cells are recruited at a slow rate into the differentiated subpopulation, the completion of the differentiation process itself in the individual cell being a relatively rapid event; or (3) some combination of these two extremes. To explore this question, we have found it useful to develop a method for separating transporting from nontransporting cells at various stages of differentiation of the population.

The principle behind the separation is very simple. Cells are suspended in isotonic solutions containing Percoll and AMG. The cells that are transport-competent take up AMG and osmotically obligated water and thus swell and become less dense. Centrifugation of the Percoll-containing suspension leads to a density gradient in the suspending medium with the nontransporting cells accumulating at their isopycnic level and the transporting cells accumulating at a correspondingly lighter density in the gradient. In practice, several problems had to be resolved. First, since the parent line appeared morphologically heterogeneous, we derived several subclones by a limiting dilution technique, and the experiments were carried out with one of these designated subclone 4. Next, we showed that neither suspending the cells by trypsinization nor the presence of Percoll had a significant effect on the transport capacity of the cells at any stage of differentiation (6,7). Our suspensions retain their transport capacity undiminished for hours.

A third problem was that these cells are very efficient at regulating their volume and, thus, their density. Volume regulation in the face of cell swelling is commonly accomplished by the activation of a KCl-loss pathway (8), and a concomitant isoosmotic loss of water. We avoided the effect by depleting the cells of these ions. We suspended the cells in a buffered isotonic Na-gluconate medium containing no K, Cl, or HCO_3. In 30 minutes the cells lost about half of their K and shrank to about two-thirds of their control volume (7). On Percoll gradients the isopycnic density for control cells in HBSS was 1.050 and in Na-gluconate medium was 1.075-1.085 gm/cc (2). The cells remained shrunken, indicating that they had lost their regulatory volume increase (RVI) capability as well. This was expected in that RVI is commonly mediated by the influx of NaCl and water (8) and there was no Cl in the suspending medium.

A fourth problem arose as a consequence of our having solved the third one. With the cells in K^+-free medium, the Na,K-ATPase could not function, the normal Na^+-electrochemical gradient was depleted (but not abolished), and hexose transport was depressed (Table I). To restore normal hexose uptake it was necessary

TABLE I
UPTAKE OF AMG IN VARIOUS MEDIA

Medium	AMG Uptake (nmol/mg protein/h)
Hanks Balanced Salt Solution (HBSS)	66 ± 8
Na-gluconate	18 ± 6
Na-gluconate + 0.1 mM ouabain	10 ± 2
Na-gluconate + 2 mM $(NH_4)_2SO_4$	57 ± 6
Na-gluconate + 2 mM $(NH_4)_2SO_4$ + 0.1 mM ouabain	14 ± 1

Cells were cultured on collagen-coated filters 6 days in 0.2 mM MIX, and pre-incubated 30 min in either HBSS (controls) or K^+-free Na-gluconate medium before assay in the same medium with or without additives plus 0.1 mM [^{14}C]AMG.

for the cells to have some means for extruding the accumulating Na^+. $NH4^+$ is an effective congener of K^+ in activating Na,K-ATPase (9) as well as Na^+ transport (10). The addition of a low concentration of $(NH_4)_2SO_4$ to the Na-gluconate medium was effective in restoring the AMG uptake to nearly control levels. The $NH4^+$-stimulated component of uptake is ouabain sensitive (Table I).

For convenience it was desirable to run the gradients at room temperature, whereas our standard uptake asasays were performed at 37°C. We found that at room temperature, although the initial 15-min uptake was only half that at

37°, at 90 min it was 95 percent. Thus in most of our gradient experiments the cells were allowed a total of 90 min to accumulate AMG.

Having established the conditions for AMG uptake without volume regulation, we proceeded to show that transport-competent cells could be separated from transport-deficient cells on a single gradient. This was accomplished as shown in Fig. 2. Weakly transporting cells were labeled with [^{3}H]leucine for 60 min

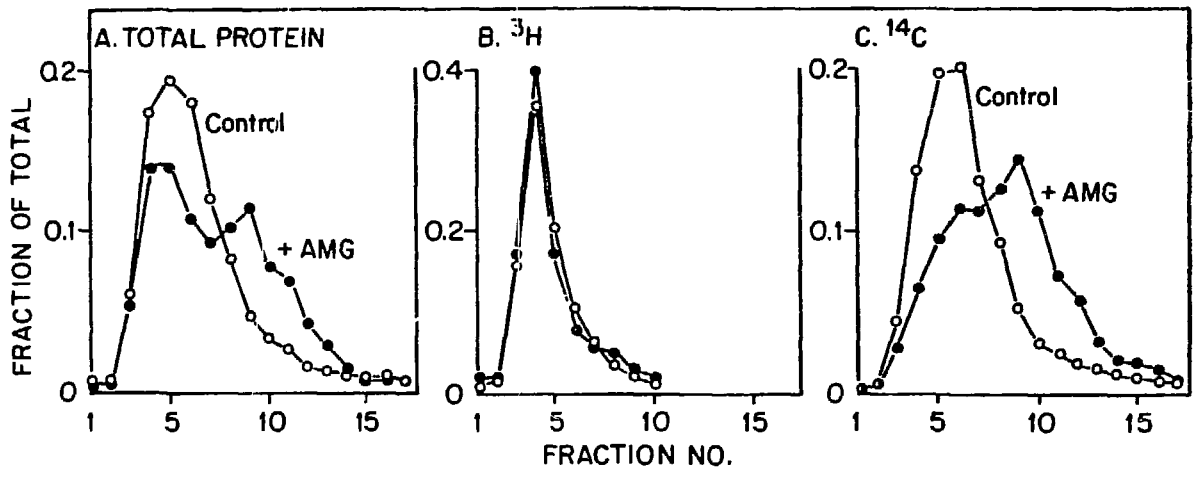

Fig. 2. Separation of transporting from nontransporting cells on Percoll gradients. Open circles, no AMG; closed circles, 5 mM AMG. Weakly transporting population ([^{3}H]) had a standard uptake of 1.5 nmol/mg protein/h; more transport competent population ([^{14}C]) had an uptake of 7.3 nmol/mg/h. Higher densities are to the left; the density difference between fraction 5 and fraction 9 is 0.012 gm/cc.

at 37°C while a population of intermediate capacity was labeled in the same way with [^{14}C]leucine. After labeling, the two populations were mixed, suspended in Na-gluconate medium with 2 mM $(NH_4)_2SO_4$ and Percoll with or without 5 mM AMG, allowed an initial 45 min to take up the hexose and centrifuged an additional 45 min. In the absence of hexose, the mixed populations sedimented as a single peak of protein, while in the presence of the hexose the protein peak was distinctly bimodal. The weakly transporting cells ([^{3}H] label) sedimented at the same density in the presence or absence of hexose. The transport-competent population ([^{14}C] label) was shifted to a lighter density in the presence of hexose with a shoulder at the density corresponding to the position of these cells in the absence of hexose. The result suggested that the uptake of hexose caused the majority, but not all, of the cells in the transporting population to swell. The transporting population appeared in fact to be comprised of two subpopulations, cells that accumulated significant quantities of AMG and cells that accumulated none.

A separate analysis confirmed that the density shift was due to AMG accumulation (Fig. 3). In this experiment cells from a transporting population were treated as above except that the gradients contained either no AMG, 1 mM [^{14}C]AMG, or 1 mM [^{14}C]AMG + 10 μM phlorizin. [Note the small peak of nonviable cells at density 1.050; they are neglected in the analyses.] Again in the presence of transported substrate the population is shifted to lighter densities than in the absence of substrate. The radioactivity in this case shows that the AMG is associated with the density-shifted cells, although it is somewhat skewed toward the control density. Phlorizin prevented both the uptake and the density shift.

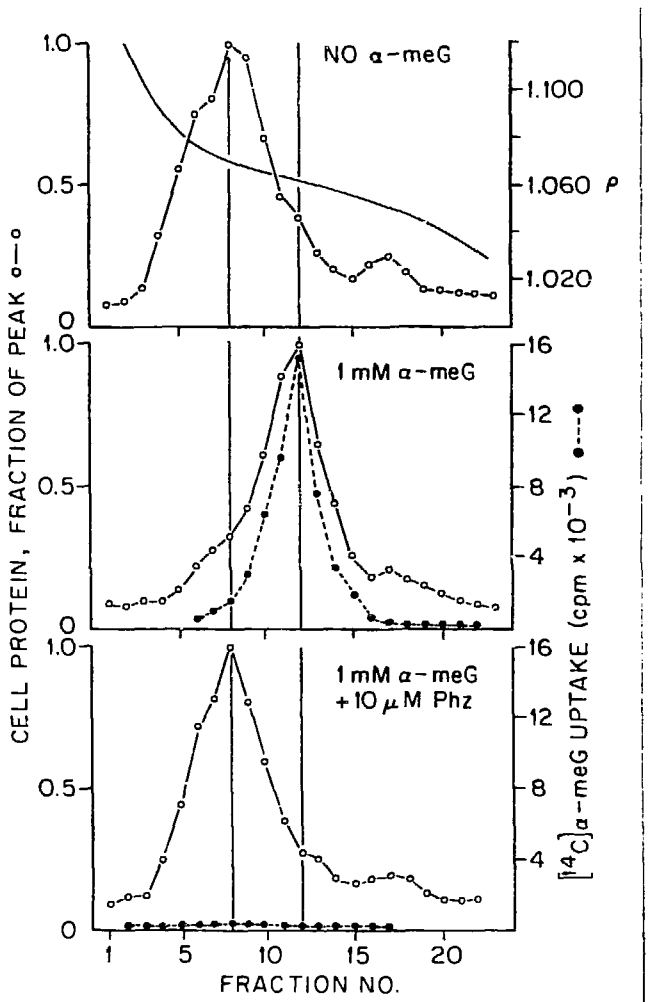

Fig. 3. Correspondence of the density shift in AMG with uptake of hexose. Cells in Na-gluconate medium, with additives as indicated on the figure. This population had been cultured in 1 mM MIX for 10 days at the time of the Percoll-gradient analysis.

The shoulder in the central panel of Fig. 3 again shows that there is a subpopulation of cells remaining at the control density, i.e., not accumulating

hexose. But it is also true that the hexose distribution as shown by the radioactivity is not symmetrically disposed under the peak of the transporting cells. The skew toward the control peak suggests that there are also cells in the population that are capable of taking up some AMG but not enough to bring them symmetrically under the "transporting" peak.

In the experiments establishing the separation methodology we tried various concentrations of AMG, from 0.1 to 5 mM, in the medium. The cell:medium concentration ratio achieved was greatest at the lowest external concentrations but the absolute differences, upon which the osmotic swelling depended, were greatest at the highest concentrations. We therefore used 5 mM in the subsequent experiments.

The following protocol was used for the developmental analysis: Cells for the entire timecourse were plated simultaneously in 100 mm dishes and allowed 2 days to attach and grow. On the second day (Day 0 of the timecourse) MIX was added to all plates at a final concentration of 1 mM. The MIX-containing medium was replaced every 2 days thereafter. On the desired days, the cells from one plate were detached and analyzed as in parts (a) and (b) of Fig. 3 with 5 mM AMG driving the density shift. At the same time an aliquot was analyzed for its 60-min uptake at 37° with AMG at 0.1 mM. The latter point was a standard that made the differentiation pattern over time for the whole population comparable to the curves shown in Fig. 1.

The analysis of the results is summarized in Table II. The data in the table are taken from series of curves with overall shapes similar to those in the upper two panels in Fig. 3, excepting that the number of cells (protein) at the

TABLE II

PERCOLL-GRADIENT ANALYSIS OF AMG-DRIVEN DENSITY SHIFTS WITH TIME IN CULTURE

Days in 1 mM MIX	C-T gm/cc	C/T in AMG	Standard uptake $[S]_i/[S]_o$
1	-	-	1.6
4	0.004	1.18	36
7	0.009	1.06	110
11	0.011	0.58	230
15	0.011	0.38	242

Conditions of the analysis as in Fig. 3 and as described in the text. C-T is the density difference between the control and "transporting" peaks, the latter identified by the peak of AMG radioactivity. C/T is the protein concentration in AMG-containing gradients at the control density relative to that at the "transporting" density. $[S]_i/[S]_o$ is concentrating capacity.

control and "transporting" densities varies systematically with time of development. In general, the following characteristics of the curves are observed: On day 1 there is virtually no uptake of AMG and no density shift. On day 4 the density shift of the cells in AMG is not discernible, but there is a small peak of radioactivity shifted about 0.005 gm/cc to the light side of the control peak. On later days the nontransporting peak becomes progressively smaller relative to the transporting peak, and the AMG-radioactivity peak in the hexose-containing gradients is always 0.009-0.011 gm/cc to the light side of the nontransporting control. At all stages of development, the radioactivity peak is skewed toward the control, nontransporting side. To restate the most important observation, the transporting peak identified by the radioactivity is always (after day 4) at the same density difference from the control. This fact suggests that the cells reaching this peak have developed their full uptake capacity and do not develop further at later times.

To describe the differentiation process we have derived a simple model that includes the following components: (1) On reaching confluence the undifferentiated population is recruited with a fixed rate constant into the differentiation pathway. (2) Since there is a measurable rate of cell renewal in this population, a growth-rate constant is included in the model. The new cells are added to the undifferentiated population. (3) Traverse of the pathway from an undifferentiated to a fully differentiated cell requires a finite time. Cells on this pathway are able to accumulate hexose to an intermediate extent between zero and the capacity characteristic of fully differentiated cells, and the intermediate capacity is linearly proportional to the cells' position in time on the pathway. The presence of this intermediate population is responsible for the skew in the AMG radioactivity distribution on the density gradients. (4) The isopycnic density of each subpopulation is distributed with a variance that is assumed to be the same for all subpopulations.

Two extremes of the model are shown in Fig. 4. In the "synchronous" form of the model, we assumed that the recruitment rate constant was very high, i.e., all cells were recruited at virtually the same time, and that the traverse of the differentiation pathway required 20 days. Twenty days was selected because this was the time required for a typical population growing in MIX to achieve a plateau of accumulation capacity. These parameters gave the expected series of unimodal symmetrical population distributions, becoming progressively less dense in 5 mM AMG on succeeding days as their hexose accumulation capacity increased. The other extreme is the "stochastic" form of the model (although both cases are in fact stochastic) in which recruitment is slow but traverse of the differentiation pathway is very rapid. In this case the undifferentiated peak at the heavier density progressively decreases as the differentiated peak increases.

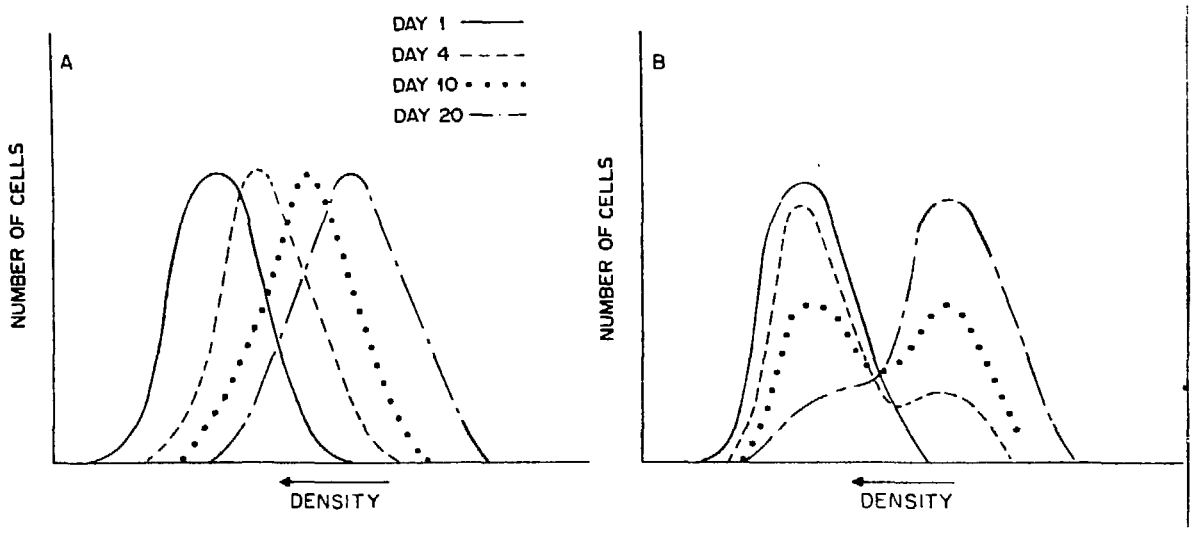

Fig. 4. (A) Synchronous model of differentiation predicts unimodal distribution of densities with progressivley less dense peaks as accumulation capacity develops. (B) Stochastic model predicts bimodal distribution of densities with the more dense "nontransporting" peak becoming progressively smaller as the lighter "transporting" peak becomes larger.

Note that the 20-day distribution in Fig. 4 closely resembles the cell distribution in the middle panel of Fig. 3, suggesting that the stochastic model is closer to reality than the synchronous model. The problem with this extreme form of the stochastic model is that the distribution of AMG radioactivity (not shown) is at all times symmetrically disposed under the peak of differentiated cells because the cells are either fully transport competent or not at all. Introduction into the model of various times for the traverse of the differentiation pathway not only gave radioactivity distributions that were skewed in the manner of the real data but in addition generated another correspondence to real data. This was that, with a variance taken from the right hand of portion of the differentiated peak in Fig. 3, the stochastic model predicted a substantial separation of the differentiated and undifferentiated subpopulations with a deep valley between them, whereas the data showed a greater overlap between the populations (cf. Fig. 2C). Incorporation of the traverse time suggested that cells in the process of differentiation might account for the cells of intermediate density.

We have used the model to derive the parameters that best fit all of the experimental data; the results suggest an intermediate pattern between the extremes. Parameters giving a satisfactory fit are: a recruitment rate of 20 percent per day from the undifferentiated subpopulation, renewal of the undifferentiated population at a rate of 7 percent per day, and a traverse time of 5–7 days from the undifferentiated to the fully differentiated cell. Since

on day 4 the accumulation capacity of the population is significant but there has not been time for the differentiating cells to reach their final state, the "transporting" peak is not displaced to the full extent (0.01 gm/cc) observed on later days. The physiological state of the cells in the traverse period remains to be established.

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SUMMARY

$LLC\text{-}PK_1$ is a clonal, morphologically polarized line of renal cells in culture. Postconfluent cells develop the Na^+-dependent capacity to transport hexoses at the their apical surface. This function is not observable during the growth phase of the cultures. To analyze the developmental process at the cellular level a method has been derived to separate transporting cells, expressing the differentiated function, from nontransporting cells. The method is based on the swelling of the cells accompanying the uptake of the nonmetabolizable glucose analog alpha methylglucoside. The swollen cells have a lower buoyant density than the undifferentiated cells and may be separated from them on density gradients. Analysis of the distribution of cells on such gradients shows that after the cells reach confluence the undifferentiated subpopulation is recruited onto the differentiation pathway with a rate constant of 0.2 per day, that 5–7 days are required for a cell to traverse this pathway to the fully differentiated state, and that once the maximum uptake capacity is achieved the cells do not develop further.