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Emerging roles for sodium dependent amino acid transport in mesenchymal cells

Review Article

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Summary. The functional aspects of sodium dependent amino acid transport in mesenchymal cells are the subject of this contribution. In a survey of the cross-talk existing among the various transport mechanisms, particular attention is devoted to the role played by substrates shared by several transport systems, such as L-glutamine. Intracellular levels of glutamine are determined by the activity of System A, the main transducer of ion gradients built on by Na,K-ATPase into neutral amino acid gradients. Changes in the activity of the System are employed to regulate intracellular amino acid pool and, hence, cell volume. System A activity has been found increased in hypertonically shrunken cells and in proliferating cells. Under both these conditions cells have to increase their volume; therefore, System A can be employed as a convenient mechanism to increase cell volume both under hypertonic and isotonic conditions. Although less well characterized, the uptake of anionic amino acids performed by System X^-_{AG} may be involved in the maintenance of intracellular amino acid pool under conditions of limited availability of neutral amino acids substrates of System A.

Keywords: Cell volume – Cell cycle – Glutamine – Glutamate

Introduction

This contribution is specifically aimed to depict some functional aspects of sodium dependent amino acid transport in cultured mammalian cells of mesenchymal origin. It describes predominantly the experimental work performed by our group on cultured human fibroblasts and NIH3T3 cells, the model cells from which most of the results presented here have been obtained. The situation depicted is quite similar to many other less well characterized mesenchymal cell models. For more complete and systematic reviews on sodium dependent amino acid transport the reader is referred to

Christensen (1990), Guidotti and Gazzola (1992), McGivan and Pastor-Anglada (1994).

In the first part of the review an overall description of the systems involved in amino acid transport is given; attention is then focused on one of these systems, the sodium-dependent, highly concentrative system A for neutral amino acids. The involvement of this system in cell volume regulation and in the progression of cell cycle is discussed on the basis of recent findings and literature data. Finally we briefly discuss possible roles played by system X^-_{AG} for anionic amino acids in the physiology of mesenchymal cells.

Amino acid traffic across the cell membrane of mesenchymal cells

The progressive characterization of amino acid transport systems has led to an increasingly complex picture of amino acid transport in mammalian cells.

In Fig. 1 the coordinated operation of some selected amino acid transport systems is shown. We shall maintain the traditional Christensen's nomenclature system (Bannai et al., 1984), since molecular characterization of several of the transport agencies described below is still lacking. It should be pointed out that each of the transport agencies indicated are likely to correspond to a family of closely related transporters. Moreover, for the sake of clarity, cationic amino acid transport through carriers of the CAT family has not been considered in Fig. 1, although this activity is probably the best characterized from a molecular point of view (see the reviews by MacLeod and by Closs in this same issue).

The description of Fig. 1 begins with system A, a secondary active transport system that operates a 1:1 symport of sodium and members of a selected

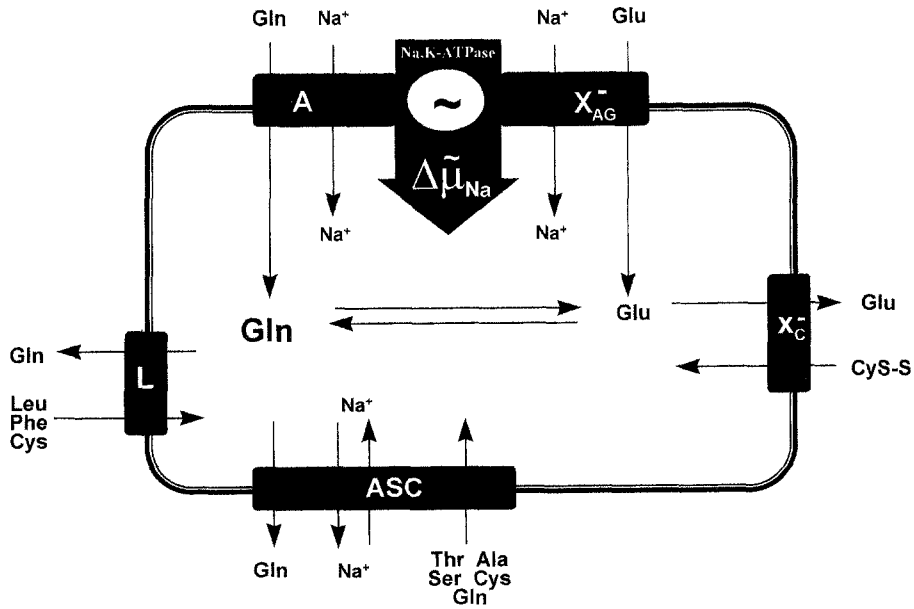

Fig. 1. A model of neutral and anionic amino acid transport in mesenchymal cells

group of neutral amino acids (such as L-proline, L-glutamine, L-alanine, L-serine) strictly dependent upon the transmembrane gradient of electrochemical potential for sodium (Gazzola et al., 1980; Dall'Asta et al., 1991); the operation of the system is therefore functionally linked to the activity of Na,K-ATPase. Thus, the accumulation of the substrates of system A can be described as the conversion of the transmembrane gradient of electrochemical potential for sodium into a transmembrane gradient of chemical potential for amino acids. Several examples of a coordinated regulation between the activities (and, presumably, the expression) of Na,K-ATPase and of system A have been described in the literature (reviewed in McGivan and Pastor-Anglada, 1994).

The operation of system A can be easily linked to that of the exchange routes characterized in several models and also in the human fibroblast (Gazzola et al., 1980; Franchi-Gazzola et al., 1982; Dall'Asta et al., 1990; Bussolati et al., 1991). These routes can be sodium-independent, as the so-called system L, or sodium-dependent, in the case of system ASC. It should be added that, while a general agreement exists about the impossibility of system L to build a significant net gradient of substrates across the membrane (Christensen, 1990; Guidotti and Gazzola, 1992), contrasting evidence exists with respect to system ASC. Indeed, data obtained in our laboratory with cultured human fibroblasts indicate that the system works predominantly by exchange (Bussolati et al., 1992). In contrast, the expression of ASC-related clones (Shafqat et al., 1993; Arriza et al., 1993) provokes a current in heterologous models (Arriza et al., 1993), implying a net flux of charges and, hence, an active nature of the transport operation performed by the system. It is not clear if these diverging results stem from the cell models employed or, rather, from the existence of closely related ASC-type carriers. We can conclude that for both systems L and ASC the demonstration of "bona fide" active nature has not yet been obtained. However, since both systems L and ASC are endowed with substrate specificities partially overlapping that of system A, the transmembrane gradient built up by the operation of system A can be somewhat redistributed among the whole large group of neutral amino acids. This means that even quasi-specific substrates of system L (i.e., leucine) or of system ASC (i.e., threonine) can reach high transmembrane distribution ratios, provided that their entry is strictly coupled to the efflux of another amino acid pre-accumulated via system A. This particular form of energizing amino acid accumulation has been defined a "tertiary active" transport process.

For tertiary active transport processes the important amino acids are those that can attain high intracellular concentrations via system A and then exchange via systems ASC and L with other amino acids. One such compound is L-glutamine that, in mesenchymal cells, exhibits a good affinity for all the three systems for neutral amino acids (Dall'Asta et al., 1990). It might be noted that we have not considered the so called system N, a transport pathway specific for glutamine, asparagine, and histidine (Kilberg et al., 1980) since no data are yet available about its bioenergetics; moreover, system N activity has been described in some (Hundal et al., 1989) but not in all mesenchymal cells

(Dall'Asta et al., 1990; Bussolati et al., 1993a). Thus, it is likely that L-glutamine is somehow at the center of the intracellular amino acid network. Interestingly, glutamine is one of the most abundant amino acids in various compartments of human body (Armstrong and Stave, 1973; Souba, 1993) as well as in most culture media. Furthermore, its intracellular concentration can reach very high values in several mammalian cells, such as in cultured human fibroblasts (Dall'Asta et al., 1994b).

This picture can be further expanded since, in the intracellular compartment, L-glutamine can be easily converted into L-glutamate and vice versa. On the other hand, the intracellular pool of L-glutamate can also be fueled by specific transport systems. One of these, system X^-_{AG} (Gazzola et al., 1981; Dall'Asta et al., 1983), is an active transport system which has the same operational features as the members of the family of Na,K-dependent glutamate carriers, cloned from nervous system and absorptive epithelia (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992). The other, system x^-_C (Bannai and Kitamura, 1980; Bannai and Kitamura, 1982; Bannai, 1986; Bussolati et al., 1986; Bussolati et al., 1993a), is an exchange, membrane potential independent, ubiquitous system, through which cystine uptake is energized by a glutamate counterflux; thus this system, under normal conditions, should not take up glutamate but, rather, dissipate the existing transmembrane gradient of the anionic amino acid.

We conclude that the establishment of high intracellular concentrations of glutamine and glutamate is a necessary condition for maintaining a large intracellular amino acid pool. The attainment of concentrations higher than 100mM primarily depends upon two transport systems, system A (through glutamine uptake) and system X^-_{AG} (through glutamate uptake). In mesenchymal cells the kinetic features of these two agencies are very different; the Vmax of system A is much higher than that of system X^-_{AG}; conversely, the affinity of system A for its substrates is much lower than that of system X^-_{AG}. Hence, mammalian cells grown in media containing high concentrations of glutamine and little glutamate achieve glutamine entry through system A. This represents the most important device for the establishment and maintenance of the intracellular amino acid pool. Several examples can be furnished to support this point. For instance, in cultured human fibroblasts cystine entry through system x^-_C depends upon extracellular glutamine, that, of course, fuels the intracellular pool of glutamate employed for the exchange (Bannai and Ishii, 1988). The intracellular pools of glutamine and glutamate can reach very high values, with concentrations that are markedly higher than the Km value of most of the enzymes that are involved in their metabolism. Thus, an obvious question arises: Why do mammalian cells maintain these high intracellular concentrations of amino acids?

Role of system A in volume regulation of mammalian cells

The field of cell volume regulation in higher eukaryotic models has seen an increasing number of studies during the last few years (see for review Sarkadi and Parker, 1991; Spring and Hoffmann, 1992; Hoffmann and Dunham, 1995).

The complex array of mechanisms through which cells restore their volume upon incubation under anisotonic conditions are collectively named RVI (Regulatory Volume Increase, for volume gain after hypertonic shrinkage) and RVD (Regulatory Volume Decrease, for volume loss after hypotonic swelling). Many cell types are not able to recover their volume efficiently when transferred directly from isotonic to hypertonic conditions (hypertonic RVI). Usually, a pretreatment in hypotonic medium (the so called RVI-post-RVD protocol) is required to somehow sensitize cells to anisotonic conditions (Sarkadi and Parker, 1991; Spring and Hoffmann, 1992; Hoffmann and Dunham, 1995). It has been suggested that the reduced intracellular chloride concentration after RVD plays a permissive role in the activation of Na, K, 2Cl cotransport in several cell types (Hoffmann and Dunham, 1995). The competence for an hypertonic RVI is strictly dependent from the availability of organic osmolytes in the extracellular compartment, at least in human fibroblasts (Gazzola et al., 1991). Indeed, after the cell shrinkage caused by hypertonic treatment, human fibroblasts can easily recover their volume in complete growth medium; on the contrary, no volume recovery is detected in serum supplemented, amino-acid-free saline solution. Again, if the hypertonic shock is performed in a complete growth medium deprived of glutamine (which represents the most abundant neutral amino acid in D-MEM), no volume recovery is observed; conversely, if glutamine is added to the saline solution a complete volume restitution is observed. The next step was to demonstrate that only neutral amino acids and methylamines substrates of system A can behave as volume-active compounds. In this context, it is of particular interest that α-(methylamino)isobutyric acid, MeAIB, the non metabolizable analogue that constitutes the most specific substrate of the system, can work as a volume-protecting compound as well as natural, fully metabolizable substrates, such as L-proline (Gazzola et al., 1991). The message stemming from these results is clear: in the volume recovery process neutral amino acids are not used as metabolites but simply as osmolytes, whose accumulation calls water into the cell.

A more complete analysis of RVI response of cultured human fibroblasts has demonstrated, on quantitative grounds, that the whole volume recovery of hypertonically shrunken human fibroblasts depends on the accumulation of amino acids, while potassium does not give a significant contribution (Dall'Asta et al., 1994c). As a whole, intracellular amino acid concentration rises by more than 60% during 4h of hypertonic treatment; most of this increase is due to changes in glutamine and glutamate. It is possible to conclude that in cultured human fibroblasts hypertonic regulatory volume increase relies entirely upon the activity of system A. This conclusion gives the proper functional context to observations describing a stimulation of system A activity and an expansion of amino acid pool in hypertonically treated avian and mammalian fibroblasts (Tramacere et al., 1984; Silvotti et al., 1991). Moreover, it is fully consistent with an increasing amount of data obtained by other groups in both epithelial (Soler et al., 1993; Ruiz-Montasell et al., 1994) and non epithelial cell models (Chen et al., 1994; Ruiz-Montasell et al., 1994; Yamauchi et al., 1994). In this context it is interesting that an analysis of

changes in intracellular osmolytes in rat tissues after hypertonic stress *in vivo* (Bedford and Leader, 1993) demonstrates changes in total content of amino acids and composition of amino acid pool that are comparable to those observed in our *in vitro* model.

While these data strongly support the general significance of the volume regulatory role of system A, they do not yet explain how the activity of the system is modulated so as to achieve an expanded intracellular amino acid pool. In cultured human fibroblasts we have identified two distinct mechanisms for the increase in system A activity involved in the volume recovery process.

During the RVI process the activity of system A, measured as amino acid influx, exhibits a 3–6-fold increase that requires 3–4 hours of incubation under hypertonic conditions for a complete development. Kinetic analysis of transport reveals that the increase is substantially dependent upon a change in Vmax of the system (Dall'Asta et al., unpublished results). This results was expected, since it has been known since several years that one of the most distinctive operative features of system A is the highly variable transport capacity in response to a number of environmental conditions, such as amino acid starvation, presence of hormones such as insulin and glucagon, cell density, proliferative status of the cell population, *in vitro* aging of the culture (discussed in the above cited reviews on amino acid transport). All these increases in the transport capacity of the system require prolonged times for their development and an active protein synthesis. Similarly, also the hypertonically induced increase in system A transport activity observed in cultured human fibroblasts requires several hours of active protein synthesis, since it is blocked by cycloheximide (Dall'Asta et al., 1994c) and by actinomycin D (Dall'Asta et al., unpublished results). The simplest explanation of this increase is an increased transcription of the gene coding for the system A carrier itself. If so, system A would resemble other organic substrates transporters whose expression is stimulated by hypertonic stress (Uchida et al., 1992; Yamauchi et al., 1992; Uchida et al., 1993; Yamauchi et al., 1993). However, evidence obtained by the group of Pastor-Anglada (presented in Ruiz-Montasell et al., 1994, and also discussed in this same issue) would indicate that the derepressed gene product is not the carrier itself but, rather, an activator of the transport process.

A careful characterization of the bioenergetics of system A in hypertonically shrunken cells has demonstrated that the enhanced accumulation of amino acids through the system is also sustained by a cell hyperpolarization that rapidly follows hypertonic shock. The characterization of the change in membrane potential has been accomplished under the same experimental conditions adopted for amino acid accumulation employing two distinct, non invasive methods for the estimation of membrane potential in adherent, intact shrunken cells. The first method employs L-arginine as a probe for membrane potential (Bussolati et al., 1987; Bussolati et al., 1989). According to this method the value of membrane potential can be obtained from the transmembrane ratio of the cationic amino acid. Some conditions have to be fulfilled in order to employ the method for reliable results: i) the

uptake of the amino acid should be due, at least for the most part, to the sodium-independent family of CAT transporters; ii) the intracellular metabolism of the amino acid should be very slow and, iii) in the particular model employed, L-arginine should not be endowed with a peculiar metabolic relevance. In human fibroblasts these requirements have been satisfied and, provided that intracellular stores of cationic amino acids have been depleted, the cationic amino acid distributes across the plasma membrane according to a typical Nernst-type relationship; thus, under these conditions, the application of Nernst relationship to the transmembrane gradient of arginine yields reliable values of the membrane potential.

More recently, we have developed a method that employs the fluorescent dye bis-oxonol in confocal microscopy. This anionic probe is used (Rink et al., 1980) for spectrofluorimetric estimation of membrane potential. We have found that confocal microscopy can be conveniently used to yield images of membrane potential changes in real time and at single cell level (Dall'Asta et al., 1994a). The depolarization caused by an increase in extracellular potassium provides a marked signal enhancement and, conversely, cell hyperpolarization obtained by activation of Na,K-ATPase is easily seen. Measurements of cell associated bis-oxonol at several extracellular potassium concentrations allows the calibration of the fluorescence signal versus the membrane potential measured with the arginine method.

With both these methods it has been possible to demonstrate that hypertonically shrunken cells rapidly hyperpolarize. Although the mechanism for this hyperpolarization is not yet clear, it is possible to determine its magnitude, that in cells incubated at an osmolality of 400mosm/kg is more than 10mV, and its duration, that in cultured human fibroblasts lasts well over 3h of incubation. The observed hyperpolarization increases the transmembrane electrochemical potential gradient for sodium, the energy source for system A (see the review by Eddy, 1992 and, for cultured human fibroblasts, Dall'Asta et al., 1991); therefore it can easily explain the increased transmembrane gradient of amino acids in RVI-exerting cells.

Given the relationship between the membrane potential and the activity of system A, one might expect a stimulation of amino acid influx. However, this is not the case; indeed, measuring the unidirectional fluxes of MeAIB 30min after the hypertonic shock (when hyperpolarization is already clearly detectable), we have found that amino acid influx is only marginally raised; this somewhat intriguing result probably derives from the shrinkage-induced rise of the intracellular concentration of transinhibiting amino acids. On the contrary, MeAIB efflux appears significantly decreased (Dall'Asta et al., 1995). If cell hyperpolarization is hampered through an increase in extracellular potassium, MeAIB efflux rises abruptly in shrunken cells. Thus, the sustained hyperpolarization maintains the efflux of neutral amino acids at low values and contributes to the increased net uptake and, hence, to the enhanced content of amino acids that is observed during the RVI response (Dall'Asta et al., manuscript in preparation). The low efflux rate for amino acids, due to cell hyperpolarization, explains why the intracellular content of these compounds

does not decrease upon hypertonic shock although their transmembrane gradient is higher in shrunken cells.

Both devices, cell hyperpolarization and synthesis-dependent increase in transport capacity, are important for volume recovery. Indeed the synthesis of new carriers or activators can be blocked with cycloheximide and hyperpolarization can be hindered performing hypertonic treatment in the presence of high extracellular potassium. In either case RVI is delayed (Dall'Asta et al., 1994c). If both hyperpolarization and protein synthesis are blocked RVI is completely abolished (Dall'Asta et al., 1994c).

In what situations of pathophysiological relevance can system A work as a volume-active transport mechanism? It is well known that cell volume changes as a result of alterations in extracellular osmolality. Actually, most of the studies performed in cell volume regulation have employed cell models highly adapted to survive marked changes in extracellular osmolality. A well characterized example is given by cells derived from the kidney medulla, in which the osmolality of the interstitium can vary widely, even under physiological conditions. If one considers other cell types, such as mesenchymal tissues, marked changes in extracellular osmolality only occur in pathological situations, such as hypernatremic or hyponatremic states or diabetic hyperglycemia. These states are summarized in Table 1 (for a more detailed discussion, see Hoffmann and Dunham, 1995). Preliminary evidence obtained in human endothelial cells would point to an increase in system A activity upon incubation in high-glucose media (Parolari et al., 1995). It is, however, evident that, although these situations can be of great clinical relevance, they hardly justify the presence of powerful volume-regulating mechanisms in mesenchymal cells that, for most of their lifespan, should face an environment whose osmolality is kept constant by specialized organ mechanisms to maintain homeostasis.

Results obtained in epithelial models have demonstrated that changes in cell volume may also derive from variations in the availability of solute substrates of active transport processes. In the hepatocyte, for instance, changes in the availability of these substrates can alter cell volume and, hence, trigger not only proper compensatory mechanisms (Kristensen, 1980;

Table 1. Selected examples of conditions associated with perturbations of cell volume

Changes in extracellular osmolarity	Changes in intracellular osmolytes
Cell swelling	
hyponatremia (water excess, Addison disease, . . .)	uptake of sugars and amino acids (liver, intestine . . .)
	exercising skeletal muscle
	ischaemic stroke (brain)
	presence of growth factors, insulin
Cell shrinkage	
hyperglycemia (diabetes)	secretion in all exocrine glands
hypernatremia (dehydration)	presence of glucagon (liver)
antidiuresis (kidney medulla)	

Kristensen and Folke, 1984) but also a number of complex cell responses (reviewed by Haussinger et al., 1993; 1994). These results led Haussinger to develop the concept of cell volume as a messenger in the regulation of cell functions. Thus, "the role of Na^+-dependent amino acid transport systems can no longer merely be identified with amino acid translocation; these transporters rather act as a transmembrane signalling system triggering cellular function by altering cellular hydration in response to substrate delivery" (Haussinger and Schliess, 1995). It is evident that, if similar conclusions can also stand for other cell models, cell volume alterations would be endowed with general significance. These concepts prompted us to reappraise the role of system A in cell proliferation.

The role of System A in cell proliferation: a reevaluation

It has been known for several years that cell proliferation is accompanied by a stimulation of transport system A activity (Guidotti and Gazzola, 1992; McGivan and Pastor-Anglada, 1994). The strongest lines of evidence discussed in these reviews are the following:

1. The activity of the system is lowered in quiescent cultured cells and stimulated when serum and growth factors are added to the culture medium.
2. The activity of the system is lowered in confluent, highly crowded cell cultures.
3. In a well established system of proliferative stimulation *in vivo*, i.e. after partial hepatectomy, the transport activity of the system peaks in the remnant liver tissue to decrease after that the proliferative activity has slowed down.
4. In many (but not all) types of tumor cells the activity of the system is high. In particular, in Ehrlich cells system A activity is maximally stimulated (i.e., it is no further stimulated by amino acid starvation).
5. *In vitro* aging of cultured human fibroblasts is associated to a progressive decrease in system A activity.

The metabolic significance of this change has been thus far quite obscure. Indeed, it requires several hours for a full development: therefore it cannot be considered a component of the early signal events in the mitogenesis. Moreover, the fact that non essential amino acids are involved as substrates of the stimulated transport process makes it highly diffcult that an increased amount of amino acids is required for metabolic reasons. Thus, in the later years, the significance of System A induction, although widely recognized, was regarded as a late metabolic event, possibly favoring an efficient protein synthesis.

On the basis of the above described role played by system A in the regulation of cell volume we suppose that an increase in the activity of the system can sustain an expansion of cell volume. This hypothesis would imply that the growth in cell size, observed during the cell cycle (Baserga, 1985; Muller et al., 1993), involves a coordinated increase of both cell dry mass and cell volume.

In support of this hypothesis we have recently demonstrated that, when a population of serum-starved NIH3T3 cells is pushed to proliferate by serum restitution, cell volume increases (Bussolati et al., in press). Actually, during the cell cycle, a cycle of volume changes occurs, with volume increasing after the entry in G_1 and returning after mitosis at values comparable to those detected in G_0 cells. The volume increase is associated with an enhanced accumulation of amino acids (Bussolati et al., in press). Among the various amino acids, glutamine and glutamate exhibit the most significant absolute and relative changes. Simultaneous measurements of transport activities of systems A and X^-_{AG}, performed in parallel, have demonstrated that, in NIH3T3 cells, neutral amino acid transport increases during the cycle while the influx of anionic compounds is markedly lowered. Thus, glutamine accumulated into the intracellular compartment (and subsequently converted partially to glutamate) probably results from an increased System A transport activity.

A model can therefore be depicted (Fig. 2) that implies that the passage from G_0 to G_1 triggers an increase in the transport activity of system A; this

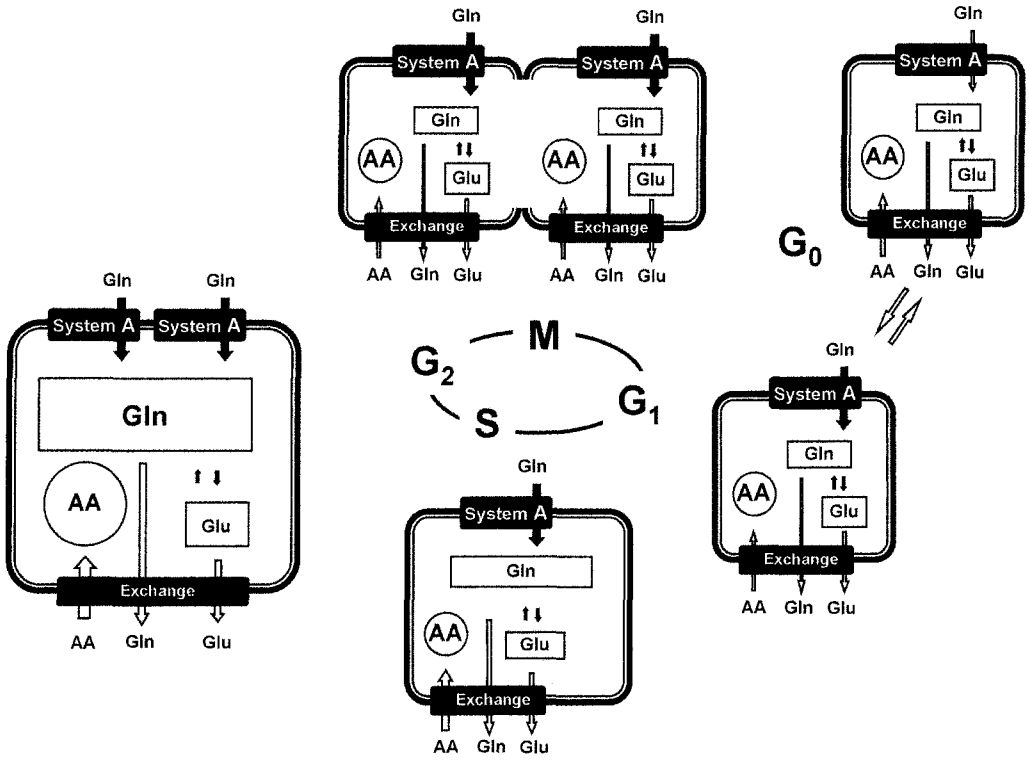

Fig. 2. Changes in amino acid transport during the cell cycle of mesenchymal cells. The increase in cell volume is explained by an increased intracellular pool of amino acids due to an enhanced transport activity for glutamine through system A. From literature data it is possible to attribute the increase of system A transport activity both to rapid mechanisms, occurring at the beginning of phase G_1 (hyperpolarization? increase in transmembrane sodium gradient?), and to a slow, protein synthesis dependent increase in the capacity of the system (synthesis of new carriers and/or activators?)

increase could be due both to rapid mechanisms such as cell hyperpolarization, and to slow responses, such as a protein synthesis dependent increase in transport capacity possibly due to an increase in the number of active carriers. As a result of the enhancement of transport activity the intracellular pools of glutamine and glutamate are raised, producing an osmotically obliged uptake of water into the cell and, therefore, an increase in cell volume; moreover, the enhanced accumulation of glutamine and glutamate lead to a generalized increase of the cell amino acid pool. This model is currently under investigation; one of its inferences would be that high concentrations of glutamine or of other system A substrates are necessary for cell proliferation not only to conduct their various metabolic roles but also to function as organic osmolytes. Actually this model works only in the presence of system A substrates in the extracellular compartment.

Halvor Christensen, discussing early attempts to define the nature of neutral amino acid transport, has stated "water movements can often be demonstrated in approximately direct relationship to solute uptake" (Christensen, 1975). These results demonstrate that, at least in the case of system A, water movements are not always a somehow untoward consequence of amino acid transport but could be, rather, the main aim of the transport process itself.

For a complete explanation of cell volume changes in cycling cells, however, one should also take into consideration the role of other transport systems that are stimulated in proliferating cells, such as the furosemide-sensitive Na,K,Cl-cotransport (Panet et al., 1982; Panet, 1985; Amsler et al., 1985; Panet et al., 1986; Paris and Poyssegur, 1986; Meyer et al., 1991) and the Na,H-exchanger (Moolenar et al., 1981; Pouyssegur et al., 1982; Schuldiner et al., 1982). Interestingly, solute influx through all these transport systems depends upon the Na^+ gradient established by Na,K-ATPase, which is also stimulated in proliferating cells (Rozengurt and Heppel, 1975; Smith and Rozengurt, 1978).

Sodium-dependent anionic amino acid transport through system X^-_{AG}: a salvage pathway?

If neutral amino acids are not present at sufficient concentrations in the extracellular medium, the maintenance of intracellular amino acid pool cannot rely on system A activity. Functional consequences can be deleterious. We are now performing experiments in which conditions of glutamine starvation are caused by treatment of cells with the antitumor enzyme asparaginase (Uggeri et al., 1995a, 1995b; Bussolati et al., 1995). If the experiment is performed in actively growing NIH3T3 cells, the intracellular concentration of glutamine rapidly falls and a significant portion of the cell population undergoes apoptotic changes (Bussolati et al., 1995).

In cells treated with L-asparaginase also the intracellular pool of glutamate also decreases markedly (Bussolati et al., 1995); this is quite surprising since the enzyme hydrolyzes glutamine to glutamate and raises impressively the extracellular concentration of the anionic amino acid. The simplest

explanation for this effect is that these cells are not able to accumulate significant amounts of glutamate due to the limited activity of system X^-_{AG} that, in actively growing NIH3T3 cells is very low (Bussolati et al., 1993b). Thus, transport activity of system X^-_{AG} may be critical for cell survival under conditions of low availability of L-glutamine.

Several recent observations demonstrate that the activity of system X^-_{AG} is strictly regulated in mesenchymal cells. Short term stimulation of the system is caused by protein kinase C activation (Franchi-Gazzola et al. 1990, 1994). On the other hand, a long term enhancement of system X^-_{AG} activity has been characterized in rat myotubes upon glutamine starvation (Low et al., 1994). Conversely, cell proliferation is associated to a marked depression of the system (Bussolati et al., 1993b); in *ras* transfected fibroblasts the expression of the oncogene, that possibly corresponds to an extreme proliferative drive, causes the complete suppression of the system (Longo et al., 1988; Uggeri et al., 1995a).

Thus, it is possible to conclude that the activity of this system is highly regulated in non nervous tissues. System X^-_{AG} is a member of the family of Na,K-dependent high affinity glutamate transporters (Hediger et al., 1995). While the role of these transport systems is well recognized in the central nervous system, since they are responsible for the re-uptake and maintenance of low levels of excitatory amino acids in the extracellular compartment, these results would indicate that also in non-nervous tissues they play important functions. One of these functions can be probably identified in maintaining the intracellular levels of glutamate under conditions at which the uptake of glutamine through system A cannot work. Under such conditions the activity of transport system X^-_{AG} (but not that of the other two systems for glutamate transport, systems ASC and x^-_C) is directly related to the intracellular levels of the anionic amino acids (Uggeri et al., 1995a). It is then known that in several cultured cell lines the adaptation to glutamine starvation is related more to the activity of sodium dependent glutamate transport (presumably through system X^-_{AG}) than to the activity of glutamine synthase (McDermott and Butler, 1993). Moreover, recent data indicate that the membrane transport of glutamate can also influence the intracellular metabolism of the anionic amino acid; in cultured kidney cells, for instance, intracellular glutaminase activity is regulated by the influx of extracellular glutamate (Welbourne and Mu, 1995).

Conclusions

Compared with the field of glucose transport, progress in amino acid transport had lagged behind, likely because of the delay in cloning the transporters. Since this constraint is fading away, amino acid transport should be now expected to know a season of exciting developments. The appreciation of the roles that these membrane functions play in several situations of pathophysiological importance could also contribute to enhance experimental interest in this area. For instance, the elucidation of the role played by sodium

dependent amino acid transport in cell volume regulation should prompt investigators to study if changes in transport activity occur in situations associated with alterations in extracellular osmolality. Moreover, a better comprehension of the involvement of changes in amino acid transport in cell cycle progression should lead to a reappraisal of the relationships existing between cell transformation and alterations of amino acid transport systems.

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References

Amsler K, Donahue JJ, Slayman CW, Adelberg EA (1985) Stimulation of bumetanide-sensitive K^+ transport in Swiss 3T3 fibroblasts by serum and mitogenic hormones. J Cell Physiol 123: 257–263

Armstrong MD, Stave U (1973) A study of plasma free amino acid levels. II. Normal values for children and adults. Metab Clin Exp 22: 561–569

Arriza JL, Kavanaugh MP, Fairman WA, Wu Y-N, Murdoch GH, North RA, Amara SG (1993) Cloning and expression of a human neutral amino acid transporter with structural similarity to the glutamate transporter gene family. J Biol Chem 268: 15329–15332

Bannai S (1986) Exchange of cystine and glutamate across plasma membrane of human fibroblasts. J Biol Chem 261: 2256–2263

Bannai S, Ishii T (1988) A novel function of glutamine in cell culture: utilization of glutamine for the uptake of cystine in human fibroblasts. J Cell Physiol 137: 360–366

Bannai S, Kitamura E (1980) Transport interaction of L-cystine and L-glutamate in human diploid fibroblasts in culture. J Biol Chem 255: 2372–2376

Bannai S, Kitamura E (1982) Adaptive enhancement of cystine and glutamate uptake in human diploid fibroblasts in culture. Biochim Biophys Acta 721: 1–10

Bannai S, Christensen HN, Vadgama JV, Ellory JC, Englesberg E, Guidotti GG, Gazzola GC, Kilberg MS, Lajtha A, Sacktor B, Sepulveda FV, Young JD, Yudilevich D, Mann G (1984) Amino acid transport systems. Nature 311: 308

Baserga R (1985) Growth in size and cell proliferation. The biology of cell reproduction. Harvard University Press, Boston, pp 103–113

Bedford JJ, Leader JP (1993) Response of tissues of the rat to anisosmolality in vivo. Am J Physiol 264 (Regulatory Integrative Comp Physiol 33): R1164–R1179

Bussolati O, Laris PC, Longo N, Dall'Asta V, Franchi-Gazzola R, Guidotti GG, Gazzola GC (1986) Effect of extracellular potassium on amino acid transport and membrane potential in fetal human fibroblasts. Biochim Biophys Acta 854: 240–250

Bussolati O, Laris PC, Nucci FA, Dall'Asta V, Longo N, Guidotti GG, Gazzola GC (1987) Dependence of L-arginine accumulation on membrane potential in cultured human fibroblasts. Am J Physiol 253 (Cell Physiol 22): C391–C397

Bussolati O, Laris PC, Nucci FA, Dall'Asta V, Franchi-Gazzola R, Guidotti GG, Gazzola GC (1989) Influx of L-arginine is an indicator of membrane potential in human fibroblasts. Am J Physiol 256 (Cell Physiol 25): C930–C935

Bussolati O, Rotoli BM, Laris PC, Dall'Asta V, Gazzola GC (1991) The preferential interaction of L-threonine with transport system ASC in cultured human fibroblasts. Biochim Biophys Acta 1070: 305–312

Bussolati O, Laris PC, Rotoli BM, Dall'Asta V, Gazzola GC (1992) Transport system ASC for neutral amino acids. An electroneutral sodium-amino acid cotransport sensitive to membrane potential. J Biol Chem 267: 8330–8335

Bussolati O, Sala R, Astorri A, Rotoli BM, Dall'Asta V, Gazzola GC (1993a) Characterization of amino acid transport in human endothelial cells. Am J Physiol 265: (Cell Physiol 34) C1006–C1014

Bussolati O, Uggeri J, Rotoli BM, Franchi-Gazzola R, Gazzola GC (1993b) The relationship between sodium-dependent transport of anionic amino acids and cell proliferation. Biochim Biophys Acta 1151: 153–160

Bussolati O, Belletti S, Uggeri J, Gatti R, Orlandini G, Dall'Asta V, Gazzola GC (1995) Characterization of apoptotic phenomena induced by treatment with L-asparaginase in NIH3T3 cells. Exp Cell Res 220: 283–291

Chen J-G, Kluss LR, Steenbergen DK, Kempson SA (1994) Hypertonic upregulation of amino acid transport system A in vascular smooth muscle cells. Am J Physiol 267 (Cell Physiol 36) C529–C536

Christensen HN (1975) Biological transport, 2nd edn. W.A. Benjamin Inc, Reading, p 16

Christensen HN (1990) Role of amino acid transport and countertransport in nutrition and metabolism. Physiol Rev 70: 43–77

Dall'Asta V, Gazzola GC, Franchi-Gazzola R, Bussolati O, Longo N, Guidotti GG (1983) Pathways of L-glutamic acid transport in cultured human fibroblasts. J Biol Chem 258: 6371–6379

Dall'Asta V, Rossi PA, Bussolati O, Guidotti GG, Gazzola GC (1990) The transport of L-glutamine into cultured human fibroblasts. Biochim Biophys Acta 1052: 106–112

Dall'Asta V, Bussolati O, Guidotti GG, Gazzola GC (1991) Energization of amino acid uptake by system A in cultured human fibroblasts. J Biol Chem 266: 1591–1596

Dall'Asta V, Gatti R, Orlandini G, Rossi PA, Bussolati O, Gazzola GC (1994a) CSLM imaging of membrane potential changes during volume perturbations of cultured human cells. FASEB J 8: A23 (Abstract)

Dall'Asta V, Rossi PA, Bussolati O, Gazzola GC (1994b) Regulatory volume decrease of cultured human fibroblasts involves changes in intracellular amino-acid pool. Biochim Biophys Acta 1220: 139–145

Dall'Asta V, Rossi PA, Bussolati O, Gazzola GC (1994c) Response of human fibroblasts to hypertonic stress. Cell shrinkage is counteracted by an enhanced active transport of neutral amino acids. J Biol Chem 269: 10485–10491

Dall'Asta V, Rossi PA, Bussolati O, Gatti R, Orlandini G, Gazzola GC (1995) Mechanisms involved in RVI-associated changes in neutral amino acid transport. FASEB J 9: A302 (Abstract)

Eddy AA (1992) Energetics of amino acid transport. In: Kilberg MS, Haussinger D (eds) Mammalian amino acid transport. Plenum Press, New York

Franchi-Gazzola R, Gazzola GC, Dall'Asta V, Guidotti GG (1982) The transport of alanine, serine, and cysteine in cultured human fibroblasts. J Biol Chem 257: 9582–9587

Franchi-Gazzola R, Bussolati O, Visigalli R, Rotoli BM, Rossi PA, Guidotti GG, Gazzola GC (1990) Phorbol esters stimulate the transport of anionic amino acids in cultured human fibroblasts. Biochem Biophys Res Commun 173: 1304–1310

Franchi-Gazzola R, Visigalli R, Bussolati O, Gazzola GC (1994) The regulation of sodium-dependent transport of anionic amino acids in cultured human fibroblasts. FEBS Lett 352: 109–112

Gazzola GC, Dall'Asta V, Guidotti GG (1980) The transport of neutral amino acids in cultured human fibroblasts. J Biol Chem 255: 929–936

Gazzola GC, Dall'Asta V, Bussolati O, Makowske M, Christensen HN (1981) A stereoselective anomaly in dicarboxylic amino acid transport. J Biol Chem 256: 6054–6059

Gazzola GC, Dall'Asta V, Nucci FA, Rossi PA, Bussolati O, Hoffmann EK, Guidotti GG (1991) Role of amino acid transport system A in the control of cell volume in cultured human fibroblasts. Cell Physiol Biochem 1: 131–142

Guidotti GG, Gazzola GC (1992) Amino acid transporters: systematic approach and principles of control. In: Kilberg MS, Haussinger D (eds) Mammalian amino acid transport. Plenum Press, New York

Haussinger D, Schliess F (1995) Cell volume and hepatocellular function. J Hepatol 22: 94–100

Haussinger D, Roth E, Lang F, Gerok W (1993) Cellular hydration state: an important determinant of protein catabolism in health and disease. Lancet 341: 1330–1332

Haussinger D, Lang F, Gerok W (1994) Regulation of cell function by the cellular hydration state. Am J Physiol 267: E343–E355

Hediger MA, Kanai Y, You G, Nussberger S (1995) Mammalian ion-coupled solute transporters. J Physiol (London) 482P: 7S–17S

Hoffmann EK, Dunham PB (1995) Membrane mechanisms and intracellular signalling in cell volume regulations. Int Rev Cytol 161: 173–262

Hundal HS, Rennie MJ, Watt PW (1987) Characteristics of L-glutamine transport in perfused rat skeletal muscle. J Physiol (London) 393: 283–305

Kanai Y, Hediger M (1992) Primary structure and functional characterization of a high-affinity glutamate transporter. Nature (London) 360: 467–471

Kilberg MS, Handlogten ME, Christensen HN (1980) Characteristics of an amino acid transport system in rat liver for glutamine, asparagine, histidine, and closely related analogs. J Biol Chem 255: 4011–4019

Kristensen LO (1980) Energization of alaine transport in isolated rat hepatocytes. Electrogenic Na^+-alanine co-transport leading to increased K^+-permeability. J Biol Chem 255: 5236–5243

Kristensen LO, Folke M (1984) Volume-regulatory K^+-efflux during concentrative uptake of alanine in isolated rat hepatocytes. Biochem J 221: 265–268

Longo N, Franchi-Gazzola R, Bussolati O, Dall'Asta V, Nucci FA, Gazzola GC (1988) Amino acid and sugar transport in mouse 3T3 cells expressing activated *ras* and *neu* oncogenes. Ann NY Acad Sci 551: 374–377

Low SY, Rennie MJ, Taylor PM (1994) Sodium-dependent glutamate transport in cultured rat myotubes increases after glutamine deprivation. FASEB J 8: 127–131

McDermott RH, Butler M (1993) Uptake of glutamate not glutamine synthetase regulates adaptation of mammalian cells to glutamine-free medium. J Cell Sci 104: 51–58

McGivan JD, Pastor-Anglada M (1994) Regulatory and molecular aspects of mammalian amino acid transport. Biochem J 299: 321–334

Meyer M, Maly K, Uberall F, Hoflacher J, Grunicke H (1991) Stimulation of K^+ transport systems by Ha-*ras*. J Biol Chem 266: 8230–8235

Moolenar WH, Mummery CL, van der Saag PT, de Laat SW (1981) Rapid ionic events and initiation of growth in serum-stimulated neuroblastoma cells. Cell 23: 789–798

Muller R, Mumberg D, Lucibello FC (1993) Signals and genes in the control of cell-cycle progression. Biochim Biophys Acta 1155: 151–179

Panet R (1985) Serum-induced net K^+ influx performed by the diuretic-sensitive transport system in quiescent NIH3T3 mouse fibroblasts. Biochim Biophys Acta 813: 141–144

Panet R, Fromer I, Atlan H (1982) Differentiation between serum stimulation of ouabain-resistant and sensitive Rb influx in quiescent NIH3T3 cells. J Membrane Biol 70: 165–169

Panet R, Amir I, Atlan H (1986) Fibroblast growth factor induces a transient net K^+ influx carried by the bumetanide-sensitive transporter in quiescent BALB/c 3T3 fibroblasts. Biochim Biophys Acta 859: 117–121

Paris S, Pouyssegur J (1986) Growth factors activate the bumetanide-sensitive $Na^+/K^+/Cl^-$ cotransport in hamster fibroblasts. J Biol Chem 261: 6177–6183

Parolari A, Sala R, Mezzadri P, Dall'Asta A, Antona C, Bussolati O, Spirito R, Gazzola GC, Biglioli P (1995) Injury of macrovascular human endothelial cells by extracellular hypertonicity. 2nd Meeting of Italian Society for Cardiovascular Research, Abs 43

Pines G, Danbolt NC, Bjoras M, Zhang Y, Bendahan A, Elde L, Koepsell H, Storm-Mathisen J, Seeberg E, Kanner BI (1992) Cloning and expression of a rat brain L-glutamate transporter. Nature (London) 360: 464–467

Pouyssegur J, Chambard JC, Franchi A, Paris S, van Obberghen-Schilung E (1982) Growth factor activation of an amiloride-sensitive Na^+/H^+ exchange system in quiescent fibroblasts: coupling to ribosomal S6 phosphorylation. Proc Natl Acad Sci USA 79: 3935–3939

Rink TJ, Montecucco C, Hesketh TR, Tsien RY (1980) Lymphocyte membrane potential assessed with fluorescent probes. Biochim Biophys Acta 595: 15–30

Rozengurt E, Heppel L (1975) Serum rapidly stimulates ouabain-sensitive $^{86}Rb^+$ influx in quiescent 3T3 cells. Proc Natl Acad Sci USA 72: 4992–4995

Ruiz-Montasell B, Cometz-Angelats M, Casado FJ, Felipe A, McGivan JD, Pastor-Anglada M (1994) Evidence for a regulatory protein involved in the increased activity of system A for neutral amino acid transport in osmotically stressed mammalian cells. Proc Natl Acad Sci USA 91: 9569–9573

Sarkadi B, Parker JC (1991) Activation of ion transport pathways by changes in cell volume. Biochim Biophys Acta 1071: 407–427

Shafqat S, Tamarappoo BK, Kilberg MS, Puranam RS, McNamara JO, Guadano-Ferraz A, Fremeau RT Jr (1993) Cloning and expression of a novel Na^+-dependent neutral amino acid transporter structurally related to mammalian Na^+/glutamate cotransporters. J Biol Chem 268: 15351–15355

Silvotti L, Petronini PG, Mazzini A, Piedimonte G, Borghetti AF (1991) Differential adaptive response to hyperosmolarity of 3T3 and transformed SV3T3 cells. Exp Cell Res 193: 253–261

Schuldiner S, Rozengurt E (1982) Na^+/H^+ antiport in Swiss 3T3 cells: stimulation leads to cytoplasmic alkalinization. Proc Natl Acad Sci USA 79: 7778–7782

Smith JB, Rozengurt E (1978) Serum stimulates the Na^+,K^+ pump in quiescent fibroblasts by increasing Na^+ entry. Proc Natl Acad Sci USA 75: 5560–5564

Soler C, Felipe A, Casado FJ, McGivan JD, Pastor-Anglada M (1993) Hyperosmolarity leads to an increase in derepressed system A activity in the renal epithelial cell line NBL-1. Biochem J 289: 653–658

Souba WW (1993) Glutamine and cancer. Ann Surg 218: 715–728

Spring KR, Hoffmann EK (1992) Cellular volume control. In: Seldin DW, Giebisch G (eds) The kidney: physiology and pathophysiology. Raven Press, New York, pp 120–152

Storck T, Schulte S, Hofmann K, Stoffel W (1992) Structure, expression, and functional analysis of a Na^+-dependent glutamate/aspartate transporter from rat brain. Proc Natl Acad Sci USA 89: 10955–10959

Tramacere M, Petronini PG, Severini A, Borghetti AF (1984) Osmoregulation of amino acid transport activity in avian fibroblasts. Exp Cell Res 151: 70–79

Uchida S, Kwon HM, Yamauchi A, Preston AS, Marumo F, Handler JS (1992) Molecular cloning of the cDNA for an MDCK cell Na^+- and Cl^--dependent taurine transporter that is regulated by hypertonicity. Proc Natl Acad Sci USA 89: 8230–8234

Uchida S, Yamauchi A, Preston AS, Kwon HM, Handler JS (1993) Medium tonicity regulates expression of the Na^+- and Cl^--dependent betaine transporter in Madin-Darby canine kidney cells by increasing transcription of the transporter gene. J Clin Invest 91: 1604–1607

Uggeri J, Belletti S, Bussolati O, Dall'Asta V, Gazzola GC (1995a) Suppression of anionic amino acid transport impairs the maintenance of intracellular glutamate in Ha-*ras*-expressing cells. Biochem Biophys Res Commun 211: 878–884

Uggeri J, Belletti S, Gatti R, Orlandini G, Bussolati O, Dall'Asta V, Gazzola GC (1995b) L-Asparaginase induces apoptosis in NIH-3T3 cells. Fund Clin Immunol 3: 84–85

Welbourne TC, Mu X (1995) Extracellular glutamate flux regulates intracellular glutaminase activity in LLC-PK1-F+ cells. Am J Physiol 268 (Cell Physiol. 37): C1418–C1424

Yamauchi A, Uchida S, Kwon HM, Preston AS, Robey RB, Garcia PA, Burg MB, Handler JS (1992) Cloning of a Na^+- and Cl^--dependent betaine transporter that is regulated by hypertonicity. J Biol Chem 267: 649–652

Yamauchi A, Uchida S, Preston AS, Kwon HM, Handler JS (1993) Hypertonicity stimulates transcription of gene for Na^+-myo-inositol cotransporter in MDCK cells. Am J Physiol 264 (Renal Fluid Electrolyte Physiol 33): F20–F23

Yamauchi A, Miyai A, Yokoyama K, Itoh T, Kamada T, Ueda N, Fujiwara Y (1994) Response to osmotic stimuli in mesangial cells: role of system A transporter. Am J Physiol 267 (Cell Physiol 36): C1493–C1500

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