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Evidence for a regulatory protein involved in the increased activity of system A for neutral amino acid transport in osmotically stressed mammalian cells

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ABSTRACT **System A for neutral amino acid transport is increased by hypertonic shock in NBL-1 cells previously induced to express system A activity by amino acid starvation. The hypertonicity-mediated effect can be blocked by cycloheximide but is insensitive to tunicamycin. The activity induced may be inactivated irreversibly by the addition of system A substrates, by a rapid mechanism insensitive to cycloheximide. In CHO-K1 cells, hypertonicity increases system A activity, as has been shown in NBL-1 cells. This effect is additive to the activity produced by derepression of system A by amino acid starvation and is insensitive to tunicamycin. Furthermore, the alanine-resistant mutant CHO-K1 ala^{r}4, which bears a mutation affecting the regulatory gene *R1*, involved in the derepression of system A activity after amino acid starvation, is still able to respond to the hypertonic shock by increasing system A activity to a level similar to that described in hypertonicity-induced derepressed CHO-K1 (wild type) cells. These results suggest (*i*) that the hypertonicity-mediated increase of system A activity occurs through a mechanism other than that involved in system A derepression and (*ii*) that a regulatory protein coded by an osmotically sensitive gene is responsible for further activation of preexisting A carriers.**

System A is the major amino acid transport system involved in the concentrative Na^+-dependent uptake of most neutral amino acids by mammalian cells. This transport system accepts methylated substrates, is trans-inhibitable, and has been found to be under nutritional and hormonal regulation in all mammalian cells so far studied (for reviews, see refs. 1 and 2). System A activity is increased by starvation for amino acids that are transported by this system. The mechanism involved has been extensively studied (3–7). Studies using somatic cell genetics led to the formulation of a model of regulation of system A, which has been extensively reviewed (2, 5, 6, 8, 9). CHO-K1 ala^{r}4 is a constitutive mutant for system A having system A activity equal to that obtained by amino acid-starved wild-type (WT) cells. ala^{r}4 cannot be further induced by amino acid starvation. Somatic cell hybrids between this mutant and the WT resulted in a normal phenotype. It was concluded that a regulatory gene, *R1*, was responsible for the repression of system A in the presence of amino acids. Total repression of system A expression by amino acids has been reported in the renal epithelial cell line NBL-1. Such cells lack system A activity unless cultured in an amino acid-free medium (10, 11). Interestingly, system A activity can be further up-regulated by hypertonicity, via a mechanism that is sensitive to cycloheximide and Colcemid (11). This suggests that system A activity is modulated by changes in cell volume.

The relevance of volume changes to cell physiology has been reviewed (2, 12). The osmoregulatory response of plasma membrane transporters after a hypertonic shock usually involves the action of ionic transporters (12), but the adaptation to long-term anisotonic conditions probably requires more permanent changes. Some cellular osmolytes such as betaine and *myo*-inositol are concentrated in renal and glia cells by a mechanism that involves *de novo* synthesis of transport proteins (13–16). This has led to the molecular cloning of two cDNAs, pSMIT and BGT1, able to induce Na^+-dependent *myo*-inositol and Na^+/Cl^--dependent betaine transport, respectively, when expressed heterologously (17, 18). Although amino acids should not be considered as osmolytes by themselves, in a few cell systems, their intracellular concentration changes abruptly in response to anisotonic stress (19, 20). As indicated above, system A seems to be under osmotic control by mechanisms that are not yet well understood.

In this study we have used the cell lines NBL-1 and CHO-K1 to further ascertain the mechanisms involved in the up-regulation of system A activity after hypertonic shock. The mutant CHO-K1 ala^{r}4 has proved particularly helpful in discriminating between the mechanism involved in system A derepression and that responsible for the osmotic-mediated response. We provide data consistent with the view that it is not system A itself that is induced after anisotonic stress, but rather, a putative system A-activating protein is responsible for the observed increase in system A activity. In summary, we postulate that regulation of system A involves a regulatory protein encoded by an osmotically sensitive gene.

MATERIALS AND METHODS

Cell Cultures, Medium, and Cultural Conditions. The bovine renal epithelial cell line NBL-1 was cultured in Ham's F-12 medium supplemented with 10% (vol/vol) newborn calf serum, 2 mM L-glutamine, and a mixture of antibiotics as reported (10, 11). CHO-K1 cells were cultured in minimum essential medium supplemented with 4% (vol/vol) fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, and a mixture of nonessential amino acids and antibiotics, as described (3). The alanine-resistant mutant ala^{r}4 was cultured in selective conditions, consisting of the same medium as the WT but with alanine and proline at 12.5 mM and 0.5 mM, respectively. Then, the cells were cultured in nonselective conditions for 24 h before the experiments.

Derepression and Hypertonicity Studies. Derepression studies were performed by culturing the cells for the indicated times in an amino acid-free medium. Basically, this consisted of the inorganic salt components of the medium, plus 10 mM

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Abbreviations: WT, wild type; MeAIB, methylaminoisobutyric acid; mosM, milliosmolar.

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glucose and 0.5% phenol red, supplemented with 0.1% bovine serum albumin and antibiotics. This medium was known to be effective in derepressing system A activity in NBL-1 cells (10, 11) and it is herein shown also to be effective in triggering the adaptive response in CHO-K1 cells.

The osmolarity of the medium, either the control or the amino acid-free medium, was increased with sucrose. We also checked that other nonpermeant osmolytes, like mannitol, are also able to exert the same effects as those induced by the disaccharide. More details about the conditions used in the experiments (i.e., incubation time, inhibitors, and addition of selected amino acids) are given in the *Results*.

Measurement of System A Activity. System A activity was measured as the fraction of 0.1 mM L-[^{3}H]alanine uptake inhibited by saturating concentrations (5 mM) of methylaminoisobutyric acid (MeAIB), the system A-specific substrate. Basically, cells were grown for the experiments in 35-mm Petri dishes. The cells were washed with 137 mM NaCl/5.4 mM KCl/1.8 mM $CaCl_2$/1.2 mM $MgSO_4$/10 mM Hepes, adjusted to pH 7.4 with Tris. Alanine metabolism was inhibited by adding 0.5 mM aminooxyacetate 20 min prior to the uptake measurements. Amino acid uptake was started by adding 1 ml of the above medium containing labeled substrate (1 μCi/ml; 1 Ci = 37 GBq). The incubation was performed at 22°C and stopped after 3 min (NBL-1 cells) or after 2 min (CHO-K1 cells) by removing the medium and washing the plates three times in 2 ml of ice-cold 137 mM NaCl/10 mM Tris·Hepes, pH 7.4. These times were shown to be suitable for measurements in initial velocity conditions (data not shown). After draining the plates, 0.5 ml of a 0.5% Triton X-100 was added. The plates were left on a rotatory shaker for 1 h and then 200 μl was removed to measure radioactivity. The rest was stored for protein determination, as described (21). System A activity was expressed as nmol of L-alanine per either 3 or 2 min per mg of protein. When adding sucrose to the medium used for the uptake measurements, no significant change was found, and thus, all the experiments were later performed without sucrose.

Since system A is known to be transinhibited by system A substrates, it was necessary to determine to what extent an amino acid-free medium could enhance system A activity by simply releasing the transporter from transinhibition. A preliminary set of experiments was performed to address this point. Either in basal conditions or after derepressing the cells or exposing them to hypertonic medium, the cells were incubated for a further hour in an amino acid-free medium in the presence of cycloheximide (10 μg/ml). Then, uptake rates were measured as indicated above. Release from transinhibition did not significantly affect system A activity in NBL-1 cells, but it induced a significant increase in transport activity in CHO-K1 cells. Thereafter, all the experiments were always performed after depleting the cells of intracellular amino acids as indicated above.

RESULTS

***De Novo*-Induced System A Activity Is Further Increased by Hypertonic Shock in NBL-1 Cells.** Further increases in amino acid transport by system A were found when derepressed NBL-1 cells (12 h in an amino acid-free medium) were grown in a hypertonic medium (200 mM sucrose) (Fig. 1). This induction was blocked by cycloheximide, an inhibitor of protein synthesis (Fig. 1*A*), but was insensitive to tunicamycin, an inhibitor of protein N-glycosylation (Fig. 1*B*). Both drugs barely modified system A activity when added to derepressed cells not exposed to sucrose treatment. To demonstrate that tunicamycin was really effective under the conditions tested, we monitored the effect of this drug on the derepression of system A activity in cells grown in an isotonic amino acid-free medium. As expected, system A induction

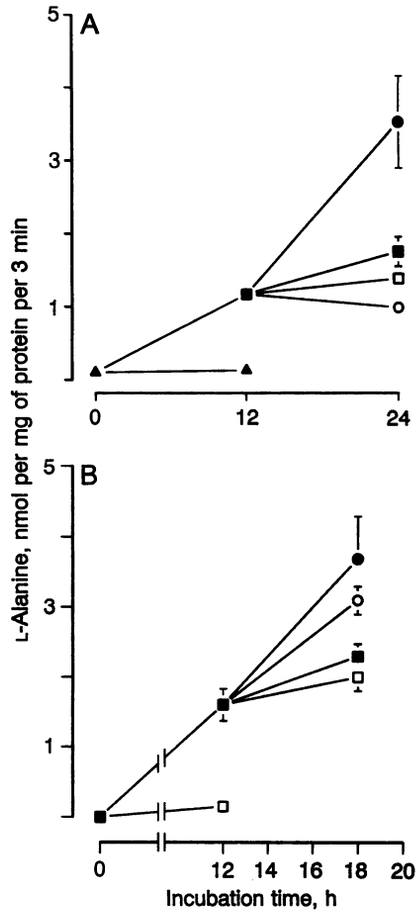

FIG. 1. Induction of system A activity by hypertonic shock in derepressed NBL-1 cells is sensitive to cycloheximide and insensitive to tunicamycin. (*A*) Confluent NBL-1 cells were cultured for 12 h either in an amino acid-free medium (■) or in a regular amino acid-containing medium (▲). Emerging system A activity was assessed for further induction by hypertonic shock. Derepressed cells were cultured for a further 12 h in the same medium in the presence (●, ○) or in the absence (■, □) of 200 mM sucrose, either with (○, □) or without (●, ■) cycloheximide (10 μg/ml). (*B*) Confluent NBL-1 cells were cultured for 12 h in an amino acid-free medium, in the presence (□) or in the absence (■) of tunicamycin (0.025 μg/ml). Then, the cells were cultured for a further 6 h in the same medium (■, □) or with 200 mM sucrose (●, ○), with (○, □) or without (●, ■) tunicamycin (0.025 μg/ml). The results are the mean ± SEM of triplicate estimations. These observations were repeated in at least four batches of cells.

triggered by amino acid starvation was blocked by tunicamycin.

Hypertonicity-Induced System A Activity in NBL-1 Cells Is Irreversibly Inactivated by Selected Amino Acids. The effect of readdition of amino acids to the cells that had previously developed induced system A activity in hypertonic amino acid-free medium was monitored (Fig. 2*A*). The addition of amino acids immediately inactivated the induced system A activity, in the presence or in the absence of sucrose. This inactivation was very rapid and irreversible, since, as indicated in the methods section, all the experiments were performed after incubating the cells for a further hour in an amino acid-free cycloheximide-supplemented medium prior to uptake measurements. This also showed that the decrease in system A activity after readdition of amino acids could not be accounted for by transinhibition due to a rise in the intracellular amino acid pool. The inactivation was completely insensitive to cycloheximide addition, at a high enough concentration of the inhibitor (10 μg/ml) to block

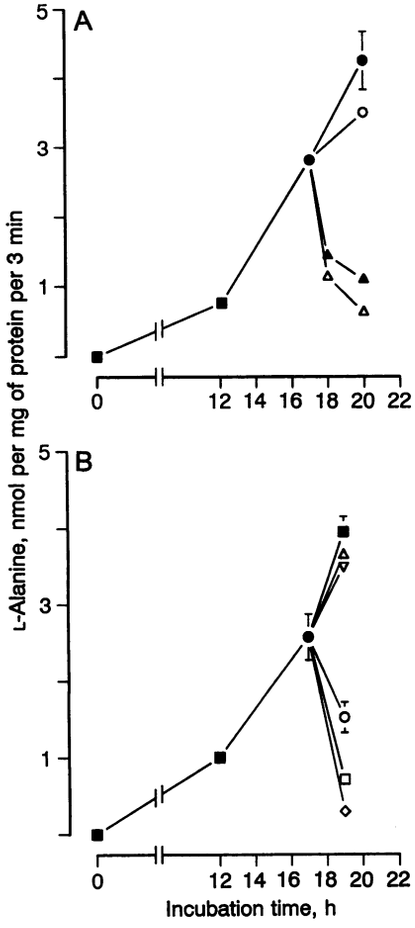

FIG. 2. Hypertonicity-induced system A activity is irreversibly inactivated by the addition of amino acids. (*A*) Confluent NBL-1 cells were derepressed for 12 h and then cultured for a further 5 h in 200 mM sucrose. At this point, cells were kept in the same amino acid-free medium, with (●) or without (○) sucrose, or in a regular amino acid-containing medium, also with (▲) or without (△) sucrose. (*B*) Confluent NBL-1 cells were derepressed for 12 h and then cultured for an additional 5 h in 200 mM sucrose. Then, single amino acids at 5 mM were added. The amino acids used were L-leucine (△), L-phenylalanine (▽), MeAIB (◇), L-glutamine (□), and L-alanine (○). Some cells were maintained in the amino acid-free medium as controls (■). The results are the mean ± SEM of triplicate estimations. These observations were repeated in at least four batches of cells.

system A derepression (data not shown). The evidence that this effect is independent of protein synthesis is consistent with the time course of the inactivation process.

To analyze the specificity of this effect, we monitored the system A-inactivating capacity of selected amino acids. Results are shown in Fig. 2*B*. Interestingly, the addition of a single system A substrate (L-alanine, L-glutamine, or MeAIB) at 5 mM inactivated induced system A activity. Other non-system A substrates, such as L-leucine and L-phenylalanine, did not exert a significant effect on hypertonicity-induced system A activity.

Hypertonicity Also Increases System A Activity in CHO-K1 Cells and the Alanine-Resistant Mutant ala^{r}4. CHO-K1 cells responded to hypertonic stress by increasing system A activity in a dose-dependent manner (Fig. 3). In this cell line, system A was expressed when cultured in basal conditions and then it was not necessary to derepress the system. The effect required a few hours (6–9 h depending on the cell batch) (data not shown) and was already significant when the basal osmolarity [around 280 milliosmolar (mosM), as measured

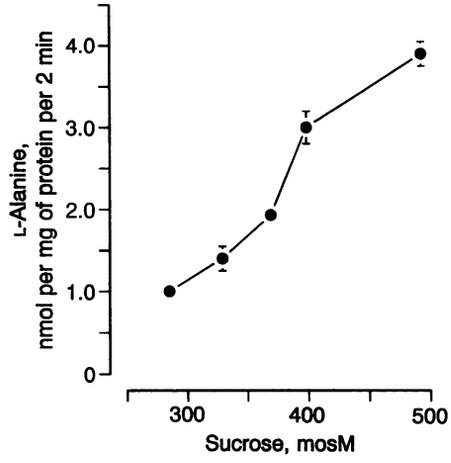

FIG. 3. Hypertonicity-mediated induction of system A activity also occurs in CHO-K1 cells and is dose dependent. Confluent CHO-K1 cells were cultured for 12 h in increasing concentrations of sucrose (50–200 mM). Osmolarity was measured with an osmometer and system A activity was determined. The results are the mean ± SEM of triplicate estimations.

directly with an osmometer] was increased to 330 mosM. Maximal induction (4-fold) was achieved at 500 mosM. To further characterize this effect, we monitored system A activity as a function of substrate concentration (0.1–10 mM L-alanine), in nontreated CHO-K1 cells or in cells exposed for 12 h to a 500 mosM medium. Kinetic parameters (mean ± SEM, $n = 4$) derived from the experimental data were K_m, 1.2 ± 0.3 and 0.6 ± 0.2 mM, and V_{max}, 46 ± 4 and 100 ± 12 nmol of L-alanine per mg of protein per 2 min ($P < 0.001$, paired t test), for cells grown in isotonic and hypertonic medium, respectively. At physiological concentrations of the substrate (0.25 mM L-alanine), the hypertonicity-induced effect was much greater than the derepression response (Table 1). Furthermore, when CHO-K1 cells were incubated for 12 h at 500 mosM and the same batch was used to monitor the derepression of system A, the two effects were found to be additive (Table 1). The mutant CHO-K1 ala^{r}4 showed basal uptake rates similar to those found in derepressed WT cells. When ala^{r}4 cells were cultured for 12 h in the same amino acid-free medium used for the WT, no significant increase in system A activity was detected, which is in agreement with the ala^{r}4 phenotype. Interestingly, when the alanine-resistant mutant was cultured for 12 h at 500 mosM, a significant increase in system A activity was found. Although when expressed as a percentage, the induction was lower (50%), the absolute uptake rates were similar to those found in derepressed hypertonicity-exposed WT cells.

The sensitivity to actinomycin D and tunicamycin of the hypertonicity-mediated induction of system A in CHO-K1 (WT) cells was also monitored (Table 1). Under conditions in which either actinomycin D (4 μg/ml) or tunicamycin (0.025 μg/ml) completely blocked the derepression of system A by amino acid starvation, only actinomycin D was able to block the hypertonicity-mediated induction.

DISCUSSION

Induction of system A activity by hypertonic shock is described in a few other systems (for review, see ref. 2). However, the mechanism(s) is not well characterized and the hypertonicity-mediated effect has somehow been related to the derepression of the system that occurred after amino acid starvation (22, 23). Results of our earlier studies (11) showed that system A activity was not expressed in NBL-1 cells exposed to hypertonic shock when cultured in Ham's F-12

Table 1. Hypertonicity-mediated induction of system A in CHO-K1 cells

Exp.	Drug or cells	System A activity, nmol of alanine per mg of protein per 2 min			
		Basal	Sucrose	Amino acid-free	Amino acid-free + sucrose
A	No inhibitors	1.5 ± 0.1	6.7 ± 0.6	3.1 ± 0.6	—
	Actinomycin D	1.3 ± 0.3	1.0 ± 0.1	1.7 ± 0.2	—
	Tunicamycin	1.3 ± 0.3	5.8 ± 0.6	1.0 ± 0.1	—
B	CHO-K1 (WT)	1.0 ± 0.1	3.1 ± 0.5	2.1 ± 0.1	4.2 ± 0.3
	CHO-K1 ala^{r}4	2.0 ± 0.1	3.9 ± 0.3	2.1 ± 0.1	ND

In experiment A, induction of system A activity after hypertonic shock is not blocked by tunicamycin. A batch of CHO-K1 cells was cultured for 12 h in Dulbecco's modified Eagle's medium (DMEM) (basal conditions), in an amino acid-free medium, or in DMEM supplemented with 200 mM sucrose, in the absence or in the presence of either actinomycin D (4 μg/ml) or tunicamycin (0.025 μg/ml). The results are the mean ± SEM of triplicate estimations. These observations were obtained with at least four batches of cells. In experiment B, the alanine resistant-mutant CHO-K1 ala^{r}4 is also sensitive to the hypertonic shock. CHO-K1 cells were cultured for 12 h in DMEM (basal conditions), in an amino acid-free medium, in DMEM supplemented with 200 mM sucrose, or in an amino acid-free medium supplemented with 200 mM sucrose. Then, system A activity was measured. The alanine-resistant mutant CHO-K1 ala^{r}4 was also cultured for 12 h in DMEM, in an amino acid-free medium, or in DMEM supplemented with 200 mM sucrose. The results are the mean ± SEM of triplicate estimations. These observations were repeated in at least four batches of cells. ND, not determined.

medium, but it was up-regulated when cells were placed in a hypertonic amino acid-free medium. This induction was specific to system A, because the major Na^+-dependent amino acid transport system in NBL-1 cells, B^0, was not up-regulated after hypertonic shock. Indeed, its activity even decreased.

In this study, we further analyzed the cellular basis involved in the up-regulation of system A activity after hypertonic shock, by using NBL-1, CHO-K1 cells, and an alanine-resistant mutant, CHO-K1 ala^{r}4, that has system A activity equal to that of the derepressed WT (that is, the WT grown under conditions of amino acid starvation). Our results are consistent with the view that system A activity is enhanced after hypertonic shock by a mechanism that does not involve the synthesis of new transporters, but rather the synthesis of a putative activating protein or group of proteins able to interact with preexisting carriers. Evidence supporting this hypothesis is detailed below. (*i*) Only derepressed NBL-1 cells, already expressing system A activity, responded to hypertonic shock by further increasing the activity of this transport system. Cells not expressing system A activity did not respond to hypertonic shock. (*ii*) Uptake rates induced by sucrose treatment seem to depend somehow on the basal activity of system A. Higher basal uptake rates resulted in higher system A activities after hypertonic shock. (*iii*) In CHO-K1 ala^{r}4 cells, hypertonicity led to transport rates similar to those found in derepressed hypertonicity-exposed CHO-K1 (WT) cells. This is consistent with the view that ala^{r}4 and derepressed CHO-K1 cells have a similar number of A carriers, and thus, for both cell lines, similar uptake rates should be expected after hypertonic shock. (*iv*) Induction of system A triggered by hypertonic shock was not sensitive to an inhibitor of N-glycosylation in either cell line NBL-1 or CHO-K1. There is much indirect evidence suggesting that system A is a glycosylated protein: system A derepression is blocked by tunicamycin (10, 24), its emergence in derepressed NBL-1 cells correlates with the incorporation of [^{3}H]mannose in a protein band in the range of 113–140 kDa (10), and its activity may be easily reconstituted after eluting proteins from isolectin or Con A columns (25). In this study we show that in both cell lines, N-glycosylation was necessary to derepress system A after amino acid starvation, but blocking N-glycosylation with tunicamycin did not inhibit the hypertonicity-mediated response in CHO-K1 and NBL-1 cells. The finding that tunicamycin did not modify basal system A activity is not a paradox, because cycloheximide did not alter basal transport activity either, which suggests that, in these cell lines and under the conditions used, the turnover time of the carrier protein is longer than the incubation time in the presence of the inhibitors. This is also in agreement with earlier studies (3). Since this effect required gene transcription (it was inhibited by actinomycin D), we suggest that hypertonic shock triggers the synthesis of a non-glycosylated protein, other than R1, by enhancing the transcription of a putative osmotically sensitive gene (Fig. 4). This activating protein may interact with the carrier itself, leading to system A activity with altered kinetic properties, as we found in this study. The finding that hypertonicity-

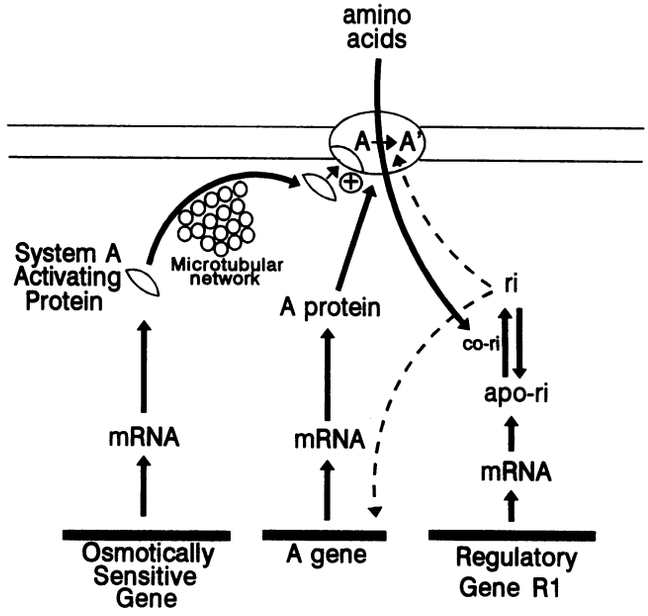

FIG. 4. Model for the regulation of system A activity in osmotically stressed cells. A model consistent with the results presented in this report and in ref. 11 is shown. According to this model, system A derepression is regulated in accordance with the Englesberg model, assuming a regulatory gene, *R1*, coding for a regulatory protein, a repressor inactivator that is inactive (Apo-RI) in the absence of amino acids. For more details we refer to previous work (3, 5, 6, 8, 9, 26). Our results are consistent with the existence of an osmotically sensitive gene coding for a regulatory protein able to interact and activate the carrier itself. Since the induction is sensitive to Colcemid, it is postulated that either the integrity of the microtubule network is necessary for system A induction or the regulatory protein itself is a cytoskeletal protein.

induced transport activity was irreversibly inactivated in NBL-1 cells, in a similar manner to that described in derepressed CHO-K1 cells (3, 4), is still consistent with the model, assuming that the sensitivity to inactivation of those A carriers synthesized after amino acid deprivation would not be modified after interacting with the "activating protein." Earlier evidence that Colcemid may block the hypertonicity-mediated effect (11) supports the view that either this regulatory protein is a cytoskeletal protein or its effect requires the integrity of the microtubule network. This aspect requires further analysis. Cytoskeletal proteins may be good candidates to interact with system A. Indeed, the concept that plasma membrane transporters may interact with cytoskeletal regulatory proteins is not new (27–29), but, to our knowledge, this is the first time that such an interaction between the cytoskeleton and an amino acid carrier has been proposed. Evidence for a direct binding between a carrier and cytoskeleton elements has been described for Na^+,K^+-ATPase. The pump may form complexes with ankyrin and fodrin in MDCK cells (30, 31). Activating and/or regulatory proteins have been reported recently for the Na^+–glucose cotransporter (32) and K^+ (33) and Cl^- (34) channels. In the latter case, an abundant cytosolic soluble protein of 40 kDa, pI_{Cln}, is the candidate for the volume-dependent activation of Cl^- channels. pI_{Cln} forms complexes with actin and other soluble proteins (34).

CHO-K1 mutants that exhibit increases in system A activity and enhanced expression of Na^+,K^+-ATPase (26, 35) overexpress the protein P1 (hsp60) (36). Genetic analysis suggests that both transporters, system A and the sodium pump, are under coordinate control by regulatory gene *R1* (26, 35). Overexpression of the three proteins may be the result of a mutation in a common regulatory element, the R1 product. It has been suggested that, at least in these cell models, P1 (hsp60) is somehow related to the increase of system A activity, by direct association to the A system transporter at the plasma membrane level or by facilitating the transport of the system A carrier complex to the plasma membrane. The recent finding that osmotic stress increases the expression of hsp70-associated proteins (37) supports the view that heat shock proteins may be involved in the hypertonicity-mediated response.

In summary, the model of regulation of system A activity by a putative activating protein is supported by experimental evidence, which otherwise bears some similarities with the effects found in other plasma membrane proteins such as ion channels and transporters. Direct assessment of these models will require the molecular identification of the carrier itself, system A, and the putative activating protein. A cDNA (SAAT1) encoding a putative system A transporter has recently been cloned (38). This cDNA is highly homologous to the Na^+–glucose cotransporter and, when transfected in COS cells, resulted in a moderate increase (2-fold) in MeAIB uptake. So far, attempts in our laboratory to detect the expression of SAAT1 mRNA in starved NBL-1 cells have proved unsuccessful.

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