The Osmoregulatory and the Amino Acid-regulated Responses of System A Are Mediated by Different Signal Transduction Pathways

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ABSTRACT The osmotic response of system A for neutral amino acid transport has been related to the adaptive response of this transport system to amino acid starvation. In a previous study (Ruiz-Montasell, B., M. Gómez-Angelats, F.J. Casado, A. Felipe, J.D. McGivan, and M. Pastor-Anglada. 1994. *Proc. Natl. Acad. Sci. USA*. 91:9569–9573), a model was proposed in which both responses were mediated by different mechanisms. The recent cloning of several isoforms of system A as well as the elucidation of a variety of signal transduction pathways involved in stress responses allow to test this model. SAT2 mRNA levels increased after amino acid deprivation but not after hyperosmotic shock. Inhibition of p38 activity or transfection with a dominant negative p38 did not alter the response to amino acid starvation but partially blocked the hypertonicity response. Inhibition of the ERK pathway resulted in full inhibition of the adaptive response of system A and no increase in SAT2 mRNA levels, without modifying the response to hyperosmolarity. Similar results were obtained after transfection with a dominant negative JNK1. The CDK2 inhibitor peptide-II decreased the osmotic response in a dose-dependent manner but did not have any effect on the adaptive response of system A. In summary, the previously proposed model of up-regulation of system A after hypertonic shock or after amino acid starvation by separate mechanisms is now confirmed and the two signal transduction pathways have been identified. The involvement of a CDK–cyclin complex in the osmotic response of system A links the activity of this transporter to the increase in cell volume previous to the entry in a new cell division cycle.

KEY WORDS: amino acid transport • MAP kinases • SAT2 • cell volume • cell cycle

INTRODUCTION

System A has been proposed to be involved in the increase in volume needed to enter a new division cycle (Bussolati et al., 1996). This change in cell volume takes place under isosmotic conditions, so osmolytes must concentrate inside the cell and neutral amino acids can play such a role (Dall'Asta et al., 1996, 1999; Gómez-Angelats et al., 1997). In fact, the reported up-regulation of system A after hypertonic treatment of the cells that leads to an increase in intracellular amino acid levels appears to be a general feature in all cell lines tested (McGivan and Pastor-Anglada, 1994; Pastor-Anglada et al., 1996). System A is not the only transporter involved in the cell cycle–induced volume change and some carriers of inorganic ions have been reported to be cell cycle regulated, such as the volume-regulated anion channel (VRAC),* which seems critical for G1/S checkpoint progression (Shen et al., 2000), or the bumetanide-sensitive Na^+, K^+, Cl^- cotransporter (Bussolati et al., 1996). However, system A must play an important role in cell-cycle progression, as deduced by the observation that in vivo N-methyl-aminoisobutiric acid (MeAIB) administration decreases liver DNA synthesis after partial hepatectomy (Freeman et al., 1999). System A may be required to provide amino acids for liver biochemical pathways and to increase cell volume, either directly providing osmolytes or substrates that can be exchanged with other amino acid transport systems (L, y^+L, ASC) that are thus contributing with new osmolytes (Bussolati et al., 2001). In fact, not only system A but also system ASC has also been reported to increase its activity after partial hepatectomy (Martínez-Mas et al., 1993).

The adaptive control of this transporter after amino acid starvation has also been characterized (Kilberg et al., 1985; Collarini and Oxender, 1987; McGivan and Pastor-Anglada, 1994) and it also implies an osmotic challenge for the cell. It is consistent with de novo synthesis of carrier proteins and appears to be transcriptionally mediated, which suggests that system A belongs

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*Abbreviations used in this paper: CDK, cyclin-dependent kinase; DSK, dual-signaling kinases; ERK, extracellular signal–regulated protein kinase; JNK, cJun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MeAIB, N-methyl-aminoisobutiric acid; MKK, MAP kinase kinase; SAT, system A transporter; 3-ATA, 3-amino-9-thio(10H)-acridone; VRAC, volume-regulated anion channel.

J. Gen. Physiol. © The Rockefeller University Press • 0022-1295/2003/07/5/12 $8.00
Volume 122 July 2003 5–16
http://www.jgp.org/cgi/doi/10.1085/jgp.200308800

to the growing list of amino acid-regulated genes (Kilberg et al., 1994; Laine et al., 1996). Hypertonicity mediates the up-regulation of system A but in a different way: although sensitive to protein synthesis inhibition, it is not sensitive to the inhibitor of N-glycosylation, tunicamycin D, in contrast to amino acid starvation (Ruiz-Montasell et al., 1994). Thus, the osmotic response is consistent with the synthesis of a regulatory protein that interacts with system A transporters at the plasma membrane (McGivan and Pastor-Anglada, 1994; Ruiz-Montasell et al., 1994; Pastor-Anglada et al., 1996), while the adaptive response was proposed to be due to an increase in system A gene expression (Ruiz-Montasell et al., 1994). Since amino acid deprivation involves a relative hypotonic shock and amino acid uptake is responsible for cell swelling even under isotonic conditions (Bussolati et al., 1996), the osmoregulatory and adaptive responses of system A may be the two sides of a similar adaptation mechanism to cell stress, with common signal transduction pathways.

The relationship between stress kinases and the molecular mechanisms involved in the transcriptional activation of several osmotic-regulated genes is controversial. JNK1, initially cloned as a component of a signal transduction pathway activated by oncoproteins and UV irradiation (Dérijard et al., 1994), was later shown to mediate osmosensing signal transduction (Galcheva-Garzova et al., 1994). Osmotic shock induces activation of several mitogen-activated protein kinase (MAPK) cascades, including extracellular signal–regulated protein kinase (ERK)1/2 and JNK (Matsuda et al., 1995). ERK1/2 have recently been shown to be involved in the adaptive response of system A to amino acid deprivation (Franchi-Gazzola et al., 1999). p38 kinase is essential for the osmotic induction of the betaine transporter gene in MDCK cells (Sheikh-Hamad et al., 1998) and both p38 and MAP kinase kinase (MKK)6 are involved in the activation of the Jak/STAT pathway after a hyperosmotic shock in COS cells (Bode et al., 1999). However, the transcriptional activation of the aldose reductase gene in PAP-HT25 is independent of p38 and JNK (Kültz et al., 1997). Using a dominant-negative JNK2 it has also been shown that hypertonicity-mediated induction of inositol uptake does not rely on this kinase in IMCD-3 cells (Wojtaszek et al., 1998).

Many features directly related to cell cycle control are regulated by the various cyclin-dependent kinase (CDK)–cyclin complexes, constituted by a catalytic CDK and a regulatory cyclin. The complexes constituted by CDK4 or CDK6 and cyclin D determine cell transit through the G1 phase while the CDK2 complexes regulate the G1/S transition (in association with cyclin E) and the S phase (in cyclin A complexes). A putative role in the G1/S transition has recently been attributed to CDK3 (Keezer and Gilbert, 2002), although many details about this CDK are still unknown. Since the increase in cell volume (and so system A activation) occurs before the entry into the S phase (Bussolati et al., 1996; Gómez-Angelats et al., 1997), the osmotic response of system A could be under the control of one of these CDK–cyclin complexes. Recently, it has been shown that CDK2–cyclin E complexes regulate other early S-phase processes such as histone gene transcription (Ma et al., 2000; Zhao et al., 2000) and $p27^{Kip1}$ degradation (Sheaff et al., 1997).

In this study, the previously proposed model of regulation of system A by amino acid depletion and hyperosmotic shock (Ruiz-Montasell et al., 1994) has been validated, since the cloning of different system A isoforms (Reimer et al., 2000; Sugawara et al., 2000; Varoqui et al., 2000; Yao et al., 2000) has allowed the identification of the isoform expressed in CHO-K1 cells, and so the variations in its mRNA levels have been analyzed. Furthermore, the signal transduction pathways involved in both responses have been determined and it is shown that both ERK and JNK are necessary for the amino acid–regulated response of system A, that is not affected by CDK–cyclin complexes. In contrast, the osmotic response of system A relies neither on ERK nor JNK, whereas the p38 kinase pathway contributes to the osmoregulatory response of the transporter, although it is not the main regulator. CDK2–cyclin complexes play an essential role in the activation of system A after a hypertonic shock, thus revealing one of the main mechanisms underlying the increase in cell volume at the beginning of the cell cycle.

MATERIALS AND METHODS

Materials

Cell culture reagents were purchased from BioWhitakker. SB202190 and PD98059 were obtained from Calbiochem. Antibodies against total or the phosphorylated forms of ERK1/2, p38, and JNK1/2 were purchased from Promega. 3-amino-9-thio(10H)-acridone (3-ATA) was obtained from Alexis Biochemicals and CDK2–cyclin inhibitory peptide II/Tat-LDL was obtained from Calbiochem. ^{2,3}H-alanine was purchased from Amersham Biosciences, ^{3}H-N-methyl-aminoisobutiric acid (MeAIB) was purchased from American Radiolabeled Chemicals and all other reagents were of analytical grade.

System A Isoform Expressed in CHO-K1 Cells and Northern Analysis

The isoform of system A expressed in CHO-K1 cells was identified by RT-PCR. Oligonucleotides were designed to specifically amplify the published rat sequences of SA1/ATA2/SAT2 (Reimer et al., 2000; Sugawara et al., 2000; Varoqui et al., 2000) and GlnT/SAT1 (Yao et al., 2000). The oligonucleotides used were: 5′-GGGAAGGTTCAACATCTCCC-3′ (positions 264–283) and 5′-GCCCATGCTTCCAATCATCACG-3′ (positions 1713–1692) for the SA1/ATA2/SAT2 sequence; and, for the GlnT/ATA1 sequence, 5′-GGAGAGGAGGAGACATTTTCAGC-3′ (positions 696–718) and 5′-GGGGGATGCTGATCAAGGAGA-3′ (positions 1560–1540). RNA was extracted from CHO-K1 cells by using the

UltraSpec II RNA isolation system (Biotecx). MMLV reverse transcriptase was from Gibco and Taq polymerase from New England Biolabs. The RT-PCR reaction consisted in an initial denaturation step at 70°C, followed by addition of the enzymes and a 1-h incubation at 42°C, 5 min at 95°C, and then 39 PCR cycles at an annealing temperature of 65°C (SAT2) or 56°C (GlnT).

Only the first set of primers produced a band which, after subcloning in pGEM-T Easy (Promega) and sequencing, was used for Northern blot assays. Sequencing was performed by the automated dideoxy termination method, at the Serveis Científico-Tècnics facilities at the University of Barcelona. Northern assays were performed on 20 μg of total RNA extracted from CHO-K1 cells cultured at the different conditions (Brown and Mackey, 1997). A ribosomal L32 cDNA probe was also used as a loading and transfer control. Densitometry of scanned films was performed by using the Phoretix Software and intensity of SAT2 bands was always referred to the intensity of L32 control bands.

Cell Lines, Culture, and Transfection

Chinese hamster ovary cells (CHO-K1) were cultured in minimum essential medium supplemented with 4% (vol/vol) fetal calf serum, 1 mM sodium pyruvate and a mixture of nonessential amino acids and antibiotics, as described elsewhere (Qian et al., 1991; Ruiz-Montasell et al., 1994).

CHO-K1 cells were transfected, as previously described (Kingston, 1997), with either pCDNA3-FJNK1 or pCDNA3-APF. The former overexpresses the human wild-type JNK1 protein, whereas the latter corresponds to a mutated JNK1 form, in which the phosphorylation site TPY has been replaced by the APF sequence, as described elsewhere (Dérijard et al., 1995). pCDNA3 alone was used as a transfection control. Expression of JNK1 and APF were assessed by PCR (forward primer: 5′-CGGGACTTAAAGCCCAG-3′; reverse primer: 5′-CCAGCATTTTG GATAACAAATCCC-3′). JNK activity was assessed on GST-ATF2 after immunoprecipitation (donated by Dr. Isabel Fabregat) (Ventura et al., 1999).

In a parallel experiment, cells were also transfected with either pCMV5-FP38 or pCMV5-AGF, which overexpress, respectively, human p38 or a mutated form of its phosphorylation site in which the sequence TGY has been replaced by AGF (Dérijard et al., 1995).

Inducible constructs were made by using the tet-off method (Chambard and Pognonec, 1998), which consists in repressing the expression of the interest gene by the presence of a tetracyclin-derived antibiotic (off condition) and derepressing its expression (on condition) by withdrawal of the antibiotic (doxycyclin, 1 μg/ml). This method had already been used to generate human MKK3 and MKK4 constructs overexpressing either the wild-type form or a dominant negative form (with the mutations S189A/T193A for MKK3 and S221A/T225A for MKK4) (Cabane et al., 2002) and it was now employed for the p38 dominant negative. Briefly, the BglII-XbaI fragment of pCMV5-AGF was subcloned in the compatible BglII-SpeI sites of the tet-off vector, pTIS. Selection of transfectants was done by cotransfecting pUHD15.1 and pTpuro, plasmids conferring sensitivity to tetracyclin and resistance to puromycin, respectively. MKK3 and MKK4 constructs were built with a myc tag and their expression was assessed by using a comrecial anti-myc antibody (Sigma-Aldrich). All inducible constructs were transfected in CHO-K1 cells using the Fu-GENE 6 method (Promega).

Hypertonicity and Amino Acid–free Studies

Derepression of system A was achieved by culturing the cells in an amino acid–free medium, which basically consisted of the inorganic salt components of the regular medium, plus 10 mM glucose and 0.5% phenol red, supplemented with 0.1% bovine serum albumin. To generate hypertonic conditions, the osmolarity of the medium was increased from 280 mOsM to nearly 500 mOsM by adding sucrose (final concentration 200 mM), as previously reported (Pastor-Anglada et al., 1996). Cells were cultured for 9 or 12 h in either hypertonic or amino acid–free medium before determining system A transport activity, as indicated below. Inhibitors of p38 (SB202190, 1 μM) and ERK (PD98059, 10 μM) were added 30 min before starting either the osmotic or the amino acid deprivation challenges, which were then maintained for 9 h. 3-ATA (20 μM) and peptide-II (7, 10, or 20 μM) were added 4 h before starting any challenge, which were then maintained for 12 h.

Recombinant Adenovirus and Infection

The recombinant p16 adenoviral vector (Ad5RSV-p16) contains the Rous sarcoma virus promoter, the wild-type human p16 cDNA, and an SV-40 polyadenylation signal in a minigene cassette inserted into the E1-deleted region of the modified adenovirus Ad5 (Cascalló et al., 1999) and was provided by Dr. Adela Mazo. Viral stocks were propagated in 293 cells, which were derived from primary embryonal kidney cells transformed by introducing sheared fragments of Ad5 DNA. CHO-K1 cells were placed in 3.5-cm plates the day before adenovirus infection (100,000 cells per plate). Cells were washed with 1 ml PBS and infection was performed by adding the virus (50 m.o.i., in serum-free EMEM) to the cell monolayers. Cell plates were incubated at 37°C for 2 h with constant agitation. Infection was stopped by adding 1.5 ml EMEM supplemented with 10% inactivated fetal calf serum. 48 h after infection, when p16 expression is maximal, medium was changed and cells were treated with 200 mM sucrose for 9 h. p16 expression was confirmed by Western blot analysis (unpublished data).

Measurement of System A Activity

System A activity was measured as the fraction of 0.1mM L-[^{3}H]-alanine uptake inhibited by saturating concentrations of MeAIB (5 mM), as previously described (Ruiz-Montasell et al., 1994). In some experiments, system A activity was also measured as direct uptake of ^{3}H-MeAIB for 2 min, at a concentration of 50 μM and a specific activity of 5 μCi/μmol. Since system A is transinhibited by system A substrates, all uptake measurements were performed after incubating the cells for 30 min in an amino acid–free medium. Alanine uptake into CHO-K1 cells is lineal for several minutes and rates were routinely measured after 2 min of incubation and expressed as nmol/mg protein/2 min

Analysis of ERK1/2, JNK1/2, and p38 Phosphorylation

CHO-K1 cells were incubated in either a hypertonic or an amino acid–free medium, as indicated above, and then treated with Triton lysis buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 100 mM NaF, 10mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, aprotinin [2 μg/ml], leupeptin [10 μM] and AEBSF [1 mM]) at selected times for the first 30 min after addition of the hypertonic or the amino acid–free media. Extracts were then centrifuged for 10 min at 12,000 rpm and the 20-μg samples of the supernatants used for Western blot.

RESULTS

Identification of System A Isoform Expressed in CHO-K1 Cells and Northern Blot Analysis

Only the oligonucleotides designed against the rat SAT2 sequence produced a band of 707 bp in the RT-PCR reaction performed with CHO-K1 RNA. Sequence

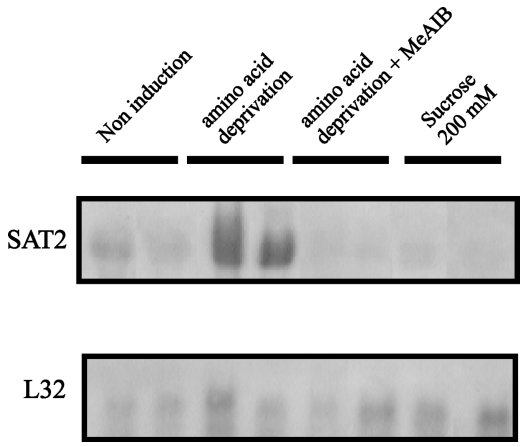

FIGURE 1. SAT2 mRNA levels in response to hypertonicity and amino acid deprivation in CHO-K1 cells. CHO-K1 cells were kept under normal culture conditions (lanes 1–2), deprived of amino acids in the medium for 6 h either in the absence (lanes 3–4) or in the presence (lanes 5–6) of 5 mM MeAIB, or treated with 200 mM sucrose (lanes 7–8). Similar results were obtained in five different samples. L32 hybridization is used as a loading and transfer control.

comparison of this band with the known sequences of system A–related genes indicated that it was the hamster counterpart of SAT2 (GenBank accession no. AF363584). The expected size was 1,450 bp, but an internal homology region in the rat sequence could be amplified with the reverse oligonucleotide, thus producing a band of 707 bp. This band was over 90% identical to the rat SAT2 sequence, and the deduced amino acid sequences between the rat and CHO counterparts were over 97% identical. So it was used for further Northern blot analysis. Fig. 1 shows the results of a Northern blot of CHO-K1 cells under normal culture conditions, under a hyperosmotic shock, or subject to amino acid deprivation. Hyperosmotic shock had no effect on system A mRNA levels, but amino acid starvation induced a 2.6-fold increase in RNA levels. The presence in the medium of a single amino acid known to block the adaptive response, MeAIB, was enough to return the RNA levels to control values.

Phosphorylation of ERK1/2, JNK1/2, and p38 in CHO-K1 Cells

To determine whether hypertonic treatment and/or amino acid starvation induce the phosphorylation of basal elements in the MAP kinase cascades, CHO-K1 cells were incubated either with 200 mM sucrose or in an amino acid–free medium and the time course of ERK1/2, JNK, and p38 phosphorylation was determined every 5 min for 30 min after the addition of medium.

Fig. 2 shows that CHO-K1 cells have fully active MAP kinase cascades that allow the differential activation of ERK, JNK, and p38 proteins in response to cell stress associated with anisotony and amino acid starvation. Hypertonicity-induced phosphorylation of ERK1/2, JNK1/3, and p38 (ERK1/2: fivefold increase after 25 min; p38 and JNK1/3: twofold increase after 10–15 min). Amino acid deprivation induced similar effects on ERK and JNK, although to a different extent (threefold) and with a different time pattern (10–15 min for ERK1/2, 10–30 min for JNK1/3). p38, however, was not phosphorylated after amino acid deprivation, which indicated that it may not participate in the adaptive response–signaling pathway.

Effect of SB202190 and PD98059 on the Osmotic and Amino Acid–regulated Responses of System A

To determine the role of p38 and ERK1/2 in the regulatory properties of system A, CHO-K1 cells were preincubated for 30 min with either 1 μM SB202190, an inhibitor of p38, or 10 μM PD98059, an inhibitor of ERKs. Specific inhibition of ERK1/2 had no effect on the response of system A to hypertonicity but the amino acid–regulated response was blocked (Table I). In accordance with the results in Fig. 1, blocking of ERK1/2 also resulted in a failure to increase SAT2 mRNA levels in response to amino acid deprivation (Fig. 3 A).

Specific inhibition of p38 resulted in a significant decrease in basal system A transport activity (30% lower than control values) (Table I). SB202190 had no effect on the relative response to amino acid starvation (about twofold), but reduced the osmoregulatory response (twofold increase versus threefold for SB202190-treated and -untreated cells respectively) (Table I). However, p38 inhibition did not block the osmotic response and CHO cells still retained the ability to increase system A activity after a hyperosmotic shock. SB202190 presence did not affect SAT2 mRNA levels in any situation (Fig. 3 C).

Role of JNK1 in System A Regulation by Hypertonicity and Amino Acid Deprivation

The role of JNK1 in system A regulation by hypertonicity and amino acid starvation was determined by generating CHO-K1 cell lines that express, in a stable manner (Fig. 4 A), either the wild-type JNK1 or a mutated form of the kinase (the APF mutant), whose ability to block endogenous JNK activity was assessed (Fig. 4 B). Overexpression of the wild-type kinase had no effect on either basal or stimulated transport activities (Table II). However transfection with the dominant negative APF mutant abolished the amino acid–regulated response, whereas transfected cells still responded to hypertonicity. These results were confirmed by direct measurement of 50 μM MeAIB uptake: in JNK1-transfected cells, amino acid starvation raised MeAIB transport rate

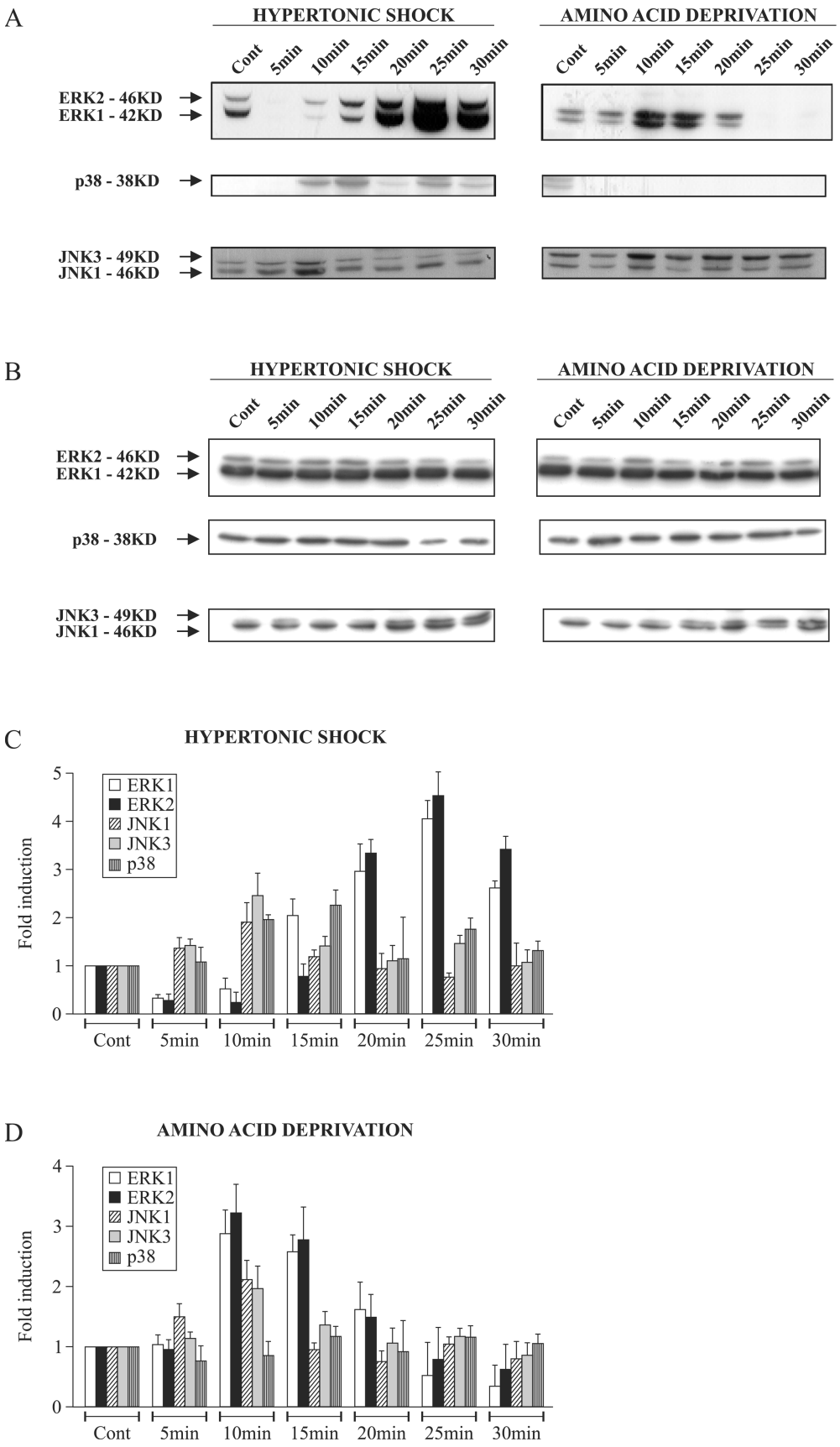

FIGURE 2. Effect of hypertonicity and amino acid starvation on ERK1/2, JNK1/2/3, and p38 phosphorylation in CHO-K1 cells. CHO-K1 cell protein extracts were prepared at different time points after hypertonic shock or amino acid starvation and analyzed by Western blot to monitor phosphorylated ERK1/2, JNK1/2/3, and p38 (A) or total ERK1/2, JNK1/2/3, and p38 (B) levels. Similar results were obtained in four independent cell cultures. The fold induction of each kinase corrected by its total levels after hypertonic shock (C) or amino acid deprivation (D) was calculated by densitometric analysis of the gels (means ± SEM of four gels).

TABLE I

Effect of PD98059 and SB202190 on the Responsiveness of System A to Hypertonicity and Amino Acid Starvation in CHO-K1 Cells

	ERK1/2 inhibition		p38 inhibition	
	No inhibitor	PD98059	No inhibitor	SB202190
No treatment	3.23 ± 0.23	2.54 ± 0.23	3.17 ± 0.34	1.79 ± 0.24†
200 mM sucrose	7.85 ± 1.00**	6.38 ± 0.92**	9.52 ± 0.34***	3.59 ± 0.83††
Amino acid deprivation	6.46 ± 0.77**	2.92 ± 0.77†	6.00 ± 0.90*	3.45 ± 0.34**†

CHO-K1 cells were preincubated in EMEM medium supplemented with 10 μM PD98059 (ERK1/2 inhibition) or with 1 μM SB202190 (p38 inhibition) for 30 min and then incubated for 9 h with no treatment or either in the presence of 200 mM sucrose or in an amino acid–free medium and system A transport activity was measured (nmol ala/2 min/mg protein). Results are the mean ± SEM of triplicate measurements made on five independent cell cultures. Statistical significance using the Student's *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, respectively, versus no treatment; †, $P < 0.05$; ††, $P < 0.01$, respectively, versus no inhibitor.

from 1.41 ± 0.32 nmol/2min/10^6 cells to 4.88 ± 1.12 nmol/2min/10^6 cells, while in APF transfected cells, rates were unaltered by amino acid deprivation (1.36 ± 0.39 nmol/2min/10^6 cells to 1.33 ± 0.51 nmol/2 min/10^6 cells, respectively). Expression of the dominant negative APF mutant resulted in lower levels of SAT2 mRNA after amino acid deprivation (Fig. 3 B).

Transfection with a Dominant Negative p38

In a parallel experiment, we tried to obtain stable transfectants expressing either the wild form of p38 or a dominant negative form (AGF mutant). As with the JNK transfection, overexpression of wild-type p38 had no effect on the adaptive or osmotic responses of the cells (Fig. 5 A). However, stable expression of the dominant negative p38 proved deleterious for CHO-K1 cells, so a new strategy was assayed. The p38 dominant negative form was expressed in CHO cells under an inducible promoter that is repressed in the presence of tetracyclin-derived antibiotics (tet-off system). The presence of doxycyclin always induced a slight, although never significant, decrease in the basal rates of system A activity. The expression of the AGF mutant induced a partial inhibition of the osmotic response, leaving the adaptive response unaffected (Fig. 5 B). MeAIB uptake followed a similar pattern: when AGF expression was repressed, cells showed a sixfold response to hyperosmotic shock (0.41 ± 0.21 nmol/2 min/10^6 cells vs. 2.45 ± 0.76 nmol/2 min/10^6 cells), but when expression was induced by antibiotic retrieval, the response was reduced to fourfold (0.37 ± 0.16 nmol/2 min/10^6 cells vs. 1.48 ± 0.33 nmol/2 min/10^6 cells).

TABLE II

Response of System A to Hypertonicity and Amino Acid Starvation in a JNK1 Negative Dominant

	Vector	JNK	APF
No treatment	3.35 ± 0.43	3.76 ± 0.34	3.01 ± 0.28
200 mM sucrose	8.69 ± 0.55***	7.99 ± 0.44***	7.06 ± 0.65**
Amino acid deprivation	6.89 ± 1.14**	7.15 ± 0.92**	3.76 ± 0.67†

CHO-K1 cells were transfected with vector alone (pCDNA3), the wild-type JNK1 (Thr183/Tyr185), and the mutated JNK1 (Ala183/Phe185, APF). Cells were incubated in EMEM (no treatment group) or in EMEM supplemented with 200 mM sucrose or in amino acid–free medium for 12 h and system A transport activity was measured (nmol ala/2 min/mg protein). Results are the mean ± SEM of triplicate measurements made on seven independent cell cultures. Statistical significance using Student's *t* test: **, $P < 0.01$; ***, $P < 0.001$, respectively, versus no treatment; †, $P < 0.05$ versus vector.

Transfection with the DSK Members MKK3 and MKK4

By using the same inducible system, we analyzed the role of two dual-signaling kinases (DSKs) upstream of JNK and p38, MKK4, and MKK3, respectively (Fig. 6). The adaptive response in the cells transfected with a dominant negative MKK4 was impaired even in the off condition, probably because the mutant is leaky and its basal expression is not zero. However, overexpression of the dominant negative MKK4 mimicked the results with JNK, blocking the adaptive but not the osmotic response. MKK3 did not reproduce the effects observed with the inhibitor SB202190 and the dominant negative p38, since overexpression of a dominant negative MKK3 had no effect on either response.

CDK Involvement in System A Osmotic Response

Since system A seems to be important in generating the increase in cell volume when the cell is entering a new division cycle, the possible involvement of CDKs in the osmotic response of system A was studied. Two separate experiments were designed to determine any possible role of CDK4–CDK6 complexes. In the first experiment, an adenovirus expressing the cDNA that encodes for the human $p16^{INK4A}$ protein was introduced in CHO-K1 cells. The $p16^{INK4A}$ protein inhibits the CDK4–CDK6:cyclin D complexes (Serrano et al., 1993). In the infection conditions used, a functional $p16^{INK4A}$ is expressed (Cascalló et al., 1999), as confirmed in our case by Western blot analysis (unpublished data). Overexpression of $p16^{INK4A}$ had no effect on the magnitude of the hyperosmotic response of system A (Table III), although a slight, nonsignificant decrease in system A activity was observed. These results were confirmed by a

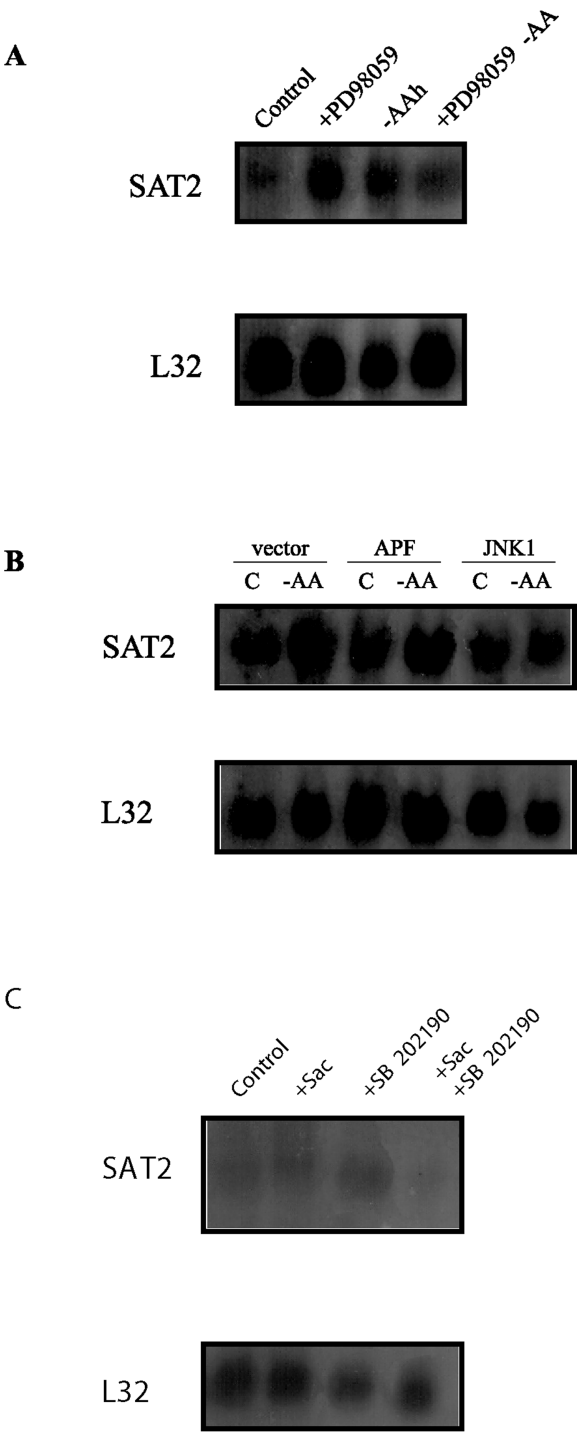

FIGURE 3. Effect of inhibition of ERK1/2, JNK1, and p38 on SAT2 mRNA levels after hypertonic shock or amino acid starvation in CHO-K1 cells. Results in Fig. 1 and Tables I and II lead to the analysis of SAT2 mRNA levels on amino acid–starved CHO-K1 cells in which ERK1/2 activity had been abolished with PD98059 (A) or JNK1 activity had been inhibited by expression of the dominant negative APF mutant (B). SAT2 mRNA levels were also analyzed on osmotically challenged CHO-K1 cells in which p38 activity had been abolished with SB202190 (C).

TABLE III

Effect of the Inhibition of CDK4,6–Cyclin D Complexes on the Osmotic Response of System A

	No treatment	200 mM sucrose
(A)		
Control	2.79 ± 0.99	4.85 ± 0.92*
Ad5RSV-p16	2.36 ± 0.51	4.73 ± 0.43*
(B)		
Control	2.76 ± 1.05	6.75 ± 0.93**
20 μM 3-ATA	1.91 ± 0.93	4.27 ± 1.24*

(A) CHO-K1 cells were infected with the (Ad5RSV-p16) adenoviral vector (50 m.o.i., in serum-free EMEM). 48 h after infection, cells were either kept in normal medium or treated with 200 mM sucrose for 12 h. (B) CHO-K1 cells were preincubated in EMEM alone or supplemented with 20 mM 3-ATA for 4 h and then incubated for 12 h with no treatment or in the presence of 200 mM sucrose. In all cases, results are the mean ± SEM of quadruplicate measurements made on four independent cell cultures. Statistical significance using the Student's *t* test: *, $P < 0.05$; **, $P < 0.01$ sucrose versus no treatment.

second experiment in which CHO-K1 cells were incubated with 3-ATA (a CDK4 specific inhibitor) (Kubo et al., 1999). Inhibition of CDK4 again reduced system A activity in the basal state, but did not affect the osmotic response of system A (2.4-fold in nontreated cells vs. 2.2-fold in 3-ATA–treated cells) (Table III). CDK2 involvement in the osmotic regulation of system A was analyzed by using its specific inhibitor peptide-II/Tat-LDL (Chen et al., 1999). Peptide-II inhibited system A osmotic response in a dose-dependent manner: with 10 μM, a 50% inhibition was achieved, whereas 20 μM induced a 90% inhibition of the osmotic response (Fig. 7). No effect of 3-ATA or peptide-II on the amino acid deprivation response were seen (unpublished data).

DISCUSSION

The neutral amino acid transport system A shows two regulatory responses that appear to be a general feature of this transporter, the adaptive response to amino acid deprivation and the response after a hypertonic shock (Kilberg et al., 1985; Collarini and Oxender, 1987; McGivan and Pastor-Anglada, 1994; Pastor-Anglada et al., 1996). The ability of system A to respond to hyperosmolarity has been related to the increase of cell volume needed by the cell before the entry in a new cell cycle (Bussolati et al., 1996), since it provides neutral amino acids that can contribute to cell volume increase either acting directly as osmolytes or indirectly as substrates for the transactivation of other amino acid transport systems, such as system L, system ASC, or system y^+L (Bussolati et al., 2001). Although the adaptive response also implies an osmotic stress for the cell, a model was put forward several years ago in which both responses were supposed to be the consequence of dif-

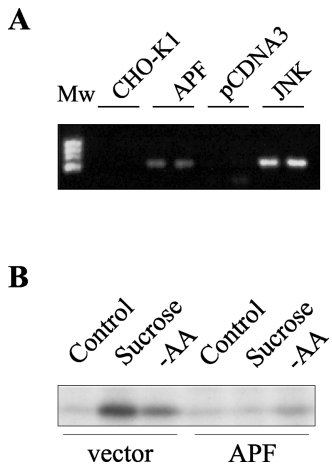

FIGURE 4. Characterization of CHO-K1 cells transfected with either JNK1 or the JNK1 dominant negative APF mutant. (A) RT-PCR from total RNA from nontransfected CHO-K1 cells or in cells transfected with either JNK1, its dominant negative APF mutant, or the vector alone (pCDNA3). (B) JNK activity was assessed in CHO-K1 cells transfected either with the vector alone or with the JNK1 dominant negative APF mutant on a GST-AF substrate, in basal conditions or 9 h after a hypertonic shock or amino acid deprivation.

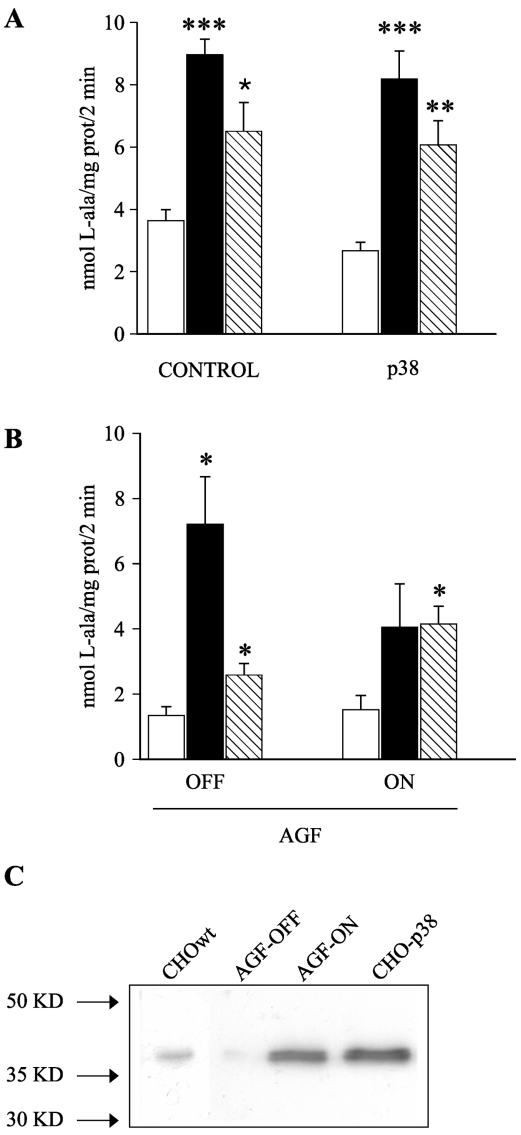

FIGURE 5. Response of system A to hypertonicity and amino acid starvation in a p38 negative dominant. (A) CHO-K1 cells were transfected with vector alone (control) or the wild-type p38 (Thr180/Tyr182). The cells were incubated in EMEM (no treatment group, open bars), in EMEM supplemented with 200 mM sucrose (solid bars), or in an amino acid–free medium (striped bars) for 12 h and system A transport activity was measured. (B) CHO-K1 cells were transfected with the mutated p38 (Ala180/Phe182, AGF). Cells were incubated in EMEM (no treatment group, open bars) or in EMEM supplemented with 200 mM sucrose (solid bars) or in amino acid–free medium (striped bars) for 12 h and system A transport activity was measured. The OFF condition is measured in the presence of 1 μg/ml doxicyclin, which represses expression of the transgene. The ON condition is measured in the absence of doxicyclin, with full expression of the transgene. Results are the mean ± SEM of triplicate measurements made on four independent cell cultures. Statistical significance using Student's t test: *, $P < 0.05$ versus no treatment. (C) Total p38 levels in nontransfected CHO-K1 cells or in cells transfected with either p38 (constitutively expressed) or its dominant negative AGF mutant (conditionally expressed, see above).

ferent cellular processes (Ruiz-Montasell et al., 1994) and recent results give support to this view (Bain et al., 2002). The adaptive control was consistent with de novo synthesis of carrier proteins while the osmotic response was consistent with the synthesis of a regulatory protein that interacts with system A transporters at the plasma membrane (Kilberg et al., 1985; Ruiz-Montasell et al., 1994; Pastor-Anglada et al., 1996). The recent cloning of several system A isoforms (Reimer et al., 2000; Sugawara et al., 2000; Varoqui et al., 2000; Yao et al., 2000) has brought the possibility of testing such a model directly. The results presented in this study show that the osmotic response has no effect on SAT2 mRNA levels, so its dependence on protein synthesis (Ruiz-Montasell et al., 1994) may be attributable to another protein (not identified yet) that acts on system A molecules. The adaptive response, in contrast, induces an increase in the levels of system A mRNA, as also stated in another recent report (Franchi-Gazzola et al., 2001), thus providing a basis for the proposed model for the regulation of system A (Ruiz-Montasell et al., 1994).

Although amino acid starvation and hypertonicity both induce osmotic phenomena in mammalian cells (Bussolati et al., 1996), the results presented in this study demonstrate that these two stress stimuli do not share common signal transduction pathways when upregulating system A in CHO-K1 cells. Kinases classically involved in osmoregulation of mammalian cells, such as JNK or ERK1/2, are necessary for the adaptive response of system A after amino acid starvation but do not contribute to the osmoregulatory response. Both JNK and ERK1/2 are rapidly phosphorylated after culturing the cells in an amino acid free or a hyperosmotic medium. Nevertheless, PD98059, an inhibitor of ERK1/2 activation, completely blocks the adaptive response and CHO-K1 cells expressing the dominant

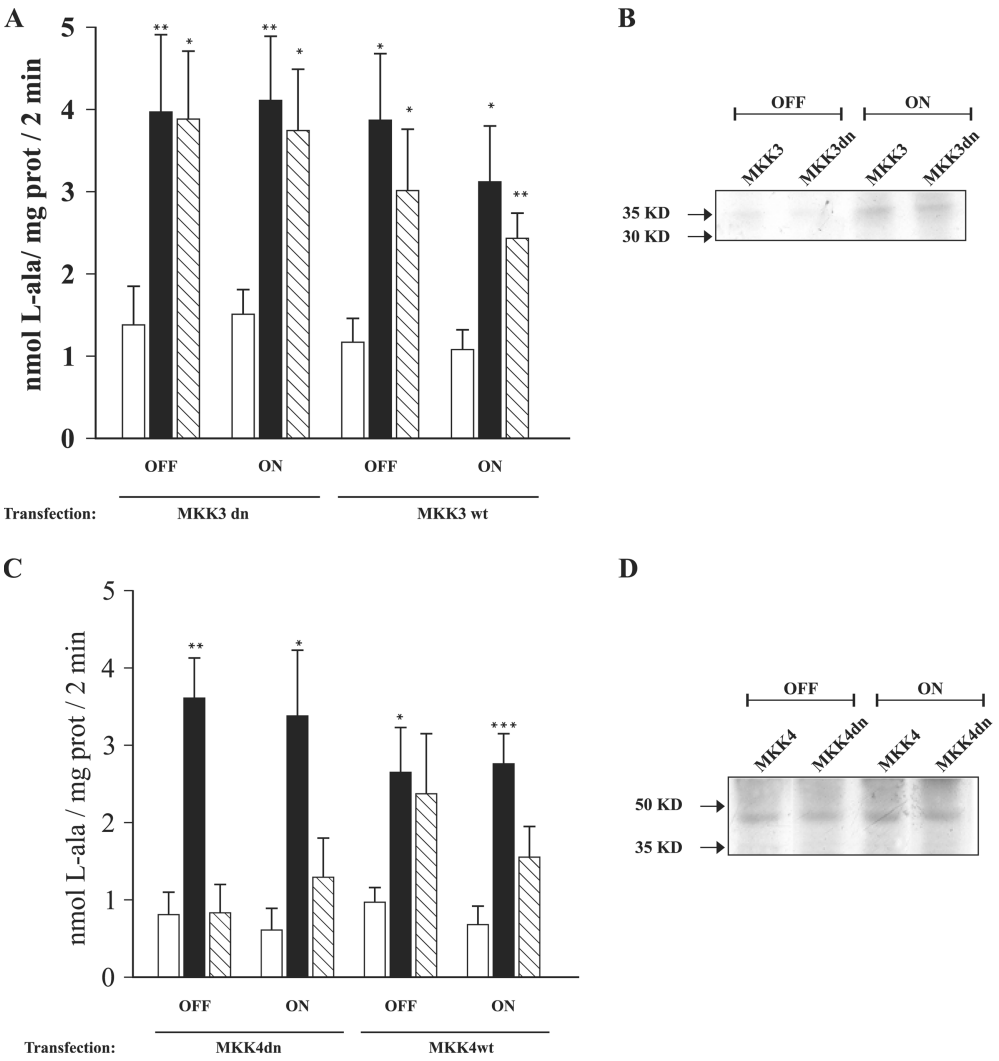

FIGURE 6. Response of system A to hypertonicity and amino acid starvation in MKK3 and MKK4 negative dominants. CHO-K1 cells were transfected with either the wild-type (wt) or the S189A/T193A mutated MKK3 (dn) (A) or with either the wild-type (wt) or the S221A/T225A mutated MKK4 (dn) (C). Cells were incubated in EMEM (no treatment group, open bars) or in EMEM supplemented with 200 mM sucrose (solid bars) or in amino acid–free medium (striped bars) for 12 h and system A transport activity was measured. The OFF and ON conditions are the same as in Fig. 5. Results are the mean ± SEM of triplicate measurements made on four independent cell cultures. Statistical significance using Student's *t* test: *, **, ***, respectively, $P < 0.05$, $P < 0.01$, and $P < 0.001$ versus no treatment. B and D show total MKK3 and MKK4 protein levels, respectively, either in the ON or the OFF conditions.

negative JNK1 APF do not up-regulate system A during amino acid starvation. Results from the dominant negative MKK4 expression confirm that JNK is involved in the adaptive response of system A. The role of ERK1/2 in the amino acid–regulated response of system A has also been demonstrated in human fibroblasts (Franchi-Gazzola et al., 1999) and it may be related to the cell shrinkage that follows amino acid starvation. Although amino acid deprivation induces a rapid cell shrinkage in CHO-K1 cells (Gómez-Angelats et al., 1997), our present results make it difficult to postulate a role for ERK1/2 in any osmotic regulatory model of system A.

The p38 cascade is activated by stress signals, but also by cytokines and growth factors (Dhanasekaran and Premkumar, 1998) that activate the DSKs MKK3/MKK6 that, in turn, phosphorylate p38 (Dérijard et al., 1995). Although MKK4 was shown to phosphorylate p38 in vitro, this study shows that the adaptive response of system A relies on the activation of JNK1, but not p38. The partial inhibition of the hypertonic response by SB202190 suggests that p38 contributes to, but is not essential for, the osmoregulatory response of system A. CHO-K1 cells transfected with a dominant negative p38 construct also showed a partial inhibition of the hyperosmotic response, but cells transfected with a dominant negative MKK3 did not, which suggests that a DSK other than MKK3 is responsible for this p38 action. The evidence that MAP kinase pathways involved in osmoprotection do not contribute to the transcriptional activation of osmotically regulated genes, such as aldose reductase (Kültz et al., 1997), the inositol transporter (Wojtaszek et al., 1998), or system A (this contribution), suggests that other pathways mediate this effect. PKC and PKA do not seem to be involved in the increase in system A transport activity after hypertonic shock, although, as for many other transporters, the basal activity is affected by PMA treatment (unpublished data). In fact, *myo*-inositol and betaine uptake in MDCK cells is inhibited by activators of PKA and PKC (Preston et al., 1995) and this effect is similar in hyper-

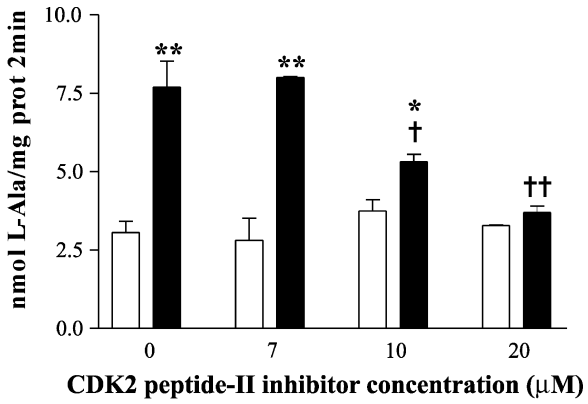

FIGURE 7. Effect of the CDK2 inhibitor Tat-LDL/peptide-II on system A activity after a hypertonic shock. CHO-K1 cells were preincubated in EMEM alone or supplemented with varying concentrations of peptide-II (7, 10, and 20 μM) for 4 h and then incubated for 12 h with no treatment (open bars) or in the presence of 200 mM sucrose (solid bars). System A transport activity was then measured. Results are the mean ± SEM of quadruplicate measurements made on 4 independent cell cultures. Statistical significance using the one-way ANOVA: P = 0.0001, effect of sucrose treatment versus control; between-group/within-group estimate F-ratio = 27.29.

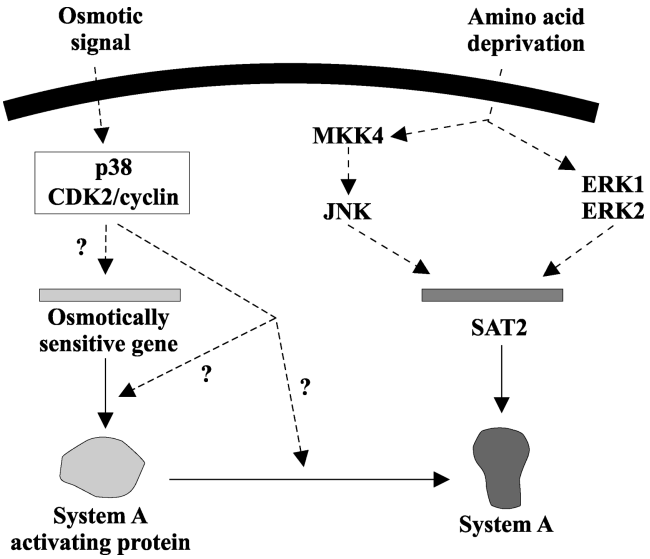

FIGURE 8. Regulatory properties of system A in CHO-K1 cells. Proposed signal transduction pathways for the adaptive and osmotic responses of system A (dashed lines). The amino acid–dependent up-regulation of system A activity relies on two independent pathways, ERK1/2 and JNK. Both are necessary for the adaptive response to occur. According to previous data (Ruiz-Montasell et al., 1994), hypertonicity triggered an increase in system A activity that was consistent with the synthesis of a putative system A–activating protein. This pathway is independent of the amino acid–deprivation signal and requires p38 and CDK2–cyclin complexes for full response, although their precise way of action is still unknown.

tonic and isotonic conditions. Thus, these kinases can mediate posttranslational regulation of the uptake of compatible osmolytes independently of the transcriptional regulation of the transporter genes. This may also apply to system A, for which the selective activation of transport activity leads to intracellular accumulation of neutral amino acids, which may also be osmolytes (Kültz and Burg, 1998). Although p38 contributes to system A osmoregulation, as for the betaine transporter (Sheikh-Hamad et al., 1998), the main transducing pathway of the osmotic regulation of system A must be a different one.

Induction of system A is one of the factors underlying the increase in volume that precedes entry into a new division cycle (Bussolati et al., 2001); this is corroborated by the correlation between system A activity and cell proliferation observed in vivo (Martínez-Mas et al., 1993; Freeman et al., 1999). CDKs regulate the main features of entry and progression in the cell cycle. CDK4-6–cyclin D complexes regulate events through the G1 phase and the G1/S transition of the cycle, while CDK2–cyclin E and CDK2–cyclin A regulate entry into the S phase and its progression (for review Roberts, 1999). CDK3, probably in association with cyclin E, has been proposed to play a role in the G1/S transition (Keezer and Gilbert, 2002), although the nature of this role is still obscure. It cannot be fully discarded that CDK3 is also involved in system A regulation, since roscovitine (an inhibitor of CDK2, CDK3, and CDK5 but not of CDK4 and CDK6) also abolished the osmotic response of system A (unpublished data) but the lack of a specific CDK3 inhibitor impairs a deeper study. The involvement of CDK2 in the hypertonic response is consistent with the knowledge that this CDK is activated when the cell is committing itself to a new cycle and is setting the conditions for the forthcoming S phase (Dorée and Galas, 1994; Draetta, 1994). Thus, among other physiological processes needed at this moment, CDK2 would also be regulating the isoosmotic increase in volume that must take place before entry into the S phase. Many of the functions of CDK2 are mediated by members of the E2F family of transcription factors (Lam and LaThangue, 1994) and the osmotic response of system A is dependent on protein synthesis (Pastor-Anglada et al., 1996), so it is tempting to postulate a role for a member of this family of transcription factors in the synthesis of this (as yet unidentified) system A regulatory protein. To our knowledge, this is the first report of a transporter related to the cell-cycle progression being controlled by CDK–cyclin complexes.

A scheme summarizing these findings is shown in Fig. 8. The signal transduction pathways involved in these two regulatory processes of system A for neutral amino acid transport in mammalian cells are different. This demonstrates that two independent mechanisms contribute to the up-regulation of system A after amino

acid starvation or hypertonic shock. The identification of CDK2 as an essential player of the osmotic regulation of system A provides a key explanation to the increase in cell volume at the beginning of the cell cycle that relies in the amino acids directly or indirectly provided by system A.

Authors thank Robin Rycroft (SAL-UB) for his editorial help.

This study was supported by grants 97/2146 (Fondo de Investigaciones Sanitarias, Ministerio de Sanidad y Consumo, Spain), SAF02-0717 (Ministerio de Ciencia y Tecnología, Spain), and 2001SGR00037 (DURSI, Generalitat de Catalunya, Spain) to M. Pastor-Anglada, PI020934 (Fondo de Investigaciones Sanitarias, Ministerio de Sanidad y Consumo, Spain) to F.J. Casado, and NCRS et ARC 9992 (France) to B. Dérijard. Marta López-Fontanals was holding a fellowship from Universitat de Barcelona and visited Benoit Dérijard's laboratory thanks to a fellowship from Service pour la Science et la Technologie (Ambassade de France).

Olaf S. Andersen served as editor.

Submitted: 15 January 2003
Revised: 3 March 2003
Accepted: 11 April 2003

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