

## Adaptive Increase of Amino Acid Transport System A Requires ERK1/2 Activation\*

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Amino acid starvation markedly stimulates the activity of system A, a widely distributed transport route for neutral amino acids. The involvement of MAPK (mitogen-activated protein kinase) pathways in this adaptive increase of transport activity was studied in cultured human fibroblasts. In these cells, a 3-fold stimulation of system A transport activity required a 6-h amino acid-free incubation. However, a rapid tyrosine phosphorylation of ERK (extracellular regulated kinase) 1 and 2, and JNK (Jun N-terminal kinase) 1, but not of p38, was observed after the substitution of complete medium with amino acid-free saline solution. ERK1/2 activity was 4-fold enhanced after a 15-min amino acid-free incubation and maintained at stimulated values thereafter. A transient, less evident stimulation of JNK1 activity was also detected, while the activity of p38 was not affected by amino acid deprivation. PD98059, an inhibitor of ERK1/2 activation, completely suppressed the adaptive increase of system A transport activity that, conversely, was unaffected by inhibitors of other transduction pathways, such as rapamycin and wortmannin, as well as by chronic treatment with phorbol esters. In the presence of either L-proline or 2-(methylaminoisobutyric) acid, two substrates of system A, the transport increase was prevented and no sustained stimulation of ERK1/2 was observed. To identify the stimulus that maintains MAPK activation, cell volume was monitored during amino acid-free incubation. It was found that amino acid deprivation caused a progressive cell shrinkage (30% after a 6-h starvation). If proline was added to amino acid-starved, shrunken cells, normal values of cell volume were rapidly restored. However, proline-dependent volume rescue was hampered if cells were pretreated with PD98059. It is concluded that (a) the triggering of adaptive increase of system A activity requires a prolonged activation of ERK1 and 2 and that (b) cell volume changes, caused by the depletion of intracellular amino acid pool, may underlie the activation of MAPKs.

System A is a highly concentrative transport route for neutral amino acids characterized by stereoselectivity, trans-inhibition (1), a strict dependence upon the transmembrane gradient of the electrochemical potential of sodium (2), and tolerance to N-methylation of substrates (3). Due to the last property,

2-(methylaminoisobutyric) acid (MeAIB),<sup>1</sup> a nonmetabolizable amino acid analogue, is the characterizing substrate of the system. Although several laboratories have tried to purify system A transporter and to clone its gene(s), the system still awaits molecular characterization (4). However, a number of studies (see Ref. 5 for review), have demonstrated that the system is a main target of several regulatory mechanisms, thus pointing to its physiological relevance. These results are consistent with data obtained *in vivo* (6).

The activity of system A markedly increases upon a prolonged incubation under amino acid-free conditions. This mechanism, named adaptive regulation or adaptive increase, was originally described in mesenchymal cells of avian origin (7), but it has also been found in many other models (5). Adaptive increase of system A transport activity is prevented by medium supplementation with a single amino acid species, such as L-proline or MeAIB. The increase in transport activity is blocked by inhibitors of transcription (8), polyadenylation (9), translation (8, 9), and protein glycosylation (10). These data can be explained by the increased synthesis of either system A transporters (8) or, alternatively, of regulatory proteins (9).

Up-regulation of system A activity has also been described upon hypertonic incubation (11–16). In cultured human fibroblasts (17) and human endothelial cells (18), the hypertonic increase in system A activity is responsible for the regulatory volume increase that follows cell shrinkage and restores cell volume. In these cell models, indeed, volume restoration is fully accounted for by the enhanced accumulation of amino acids. Amino acid-dependent cell swelling does not necessarily require hypertonic conditions since it is also observed under isotonic conditions, *i.e.* during the progression of cell cycle in mammalian mesenchymal cells (19). These studies have demonstrated that system A activity is a mechanism for cell volume control through an effective modulation of the cell content of organic compatible osmolytes.

It is known that osmotic stress triggers the activation of MAPK (mitogen-activated protein kinase) pathways (20–22). These transduction systems are important regulators of many physiological functions, including cell responses to stresses of various nature (23). Although at least three major distinct MAPK pathways (ERK, JNK, and p38) are known in mammalian cells, it is believed that each particular stimulus can activate more than one pathway simultaneously (24, 25). For in-

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<sup>1</sup> The abbreviations used are: MeAIB, 2-(methylamino)isobutyric acid; DMEM, Dulbecco's modified Eagle's medium; EBSS, Earle's balanced salt solution; ERK, extracellular regulated kinase; FBS, fetal bovine serum; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAP-kinase-2, MAPK-activated protein kinase-2; MOPS, 4-morpholine-propane sulfonic acid; PAGE, polyacrylamide gel electrophoresis; PD98059, 2'-amino-3'-methoxyflavone; PDBu, phorbol 12,13-dibutyrate; SB203580, (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole.

stance, ERK, the first MAPK characterized as a transducer of mitogenic signals, is known to correspond to a family of isozymes that are also activated by chemical or osmotic stresses (26–28). On the other hand, JNK, originally characterized as involved in stress responses, is also triggered by the interaction between epidermal growth factor and its receptor (29).

On the basis of these considerations, we undertook a study to assess the role of MAPK activation in the triggering of the adaptive increase of system A transport activity. We demonstrate here that the incubation in amino acid-free medium causes a marked activation of ERK pathway, possibly through the induction of a significant cell shrinkage.

#### EXPERIMENTAL PROCEDURES

**Cell Culture, Experimental Treatments, Amino Acid Deprivation—**Human foreskin fibroblasts were obtained from a healthy 15-year-old donor. Cells were routinely grown in 10-cm diameter dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The conditions of culture were as follows: pH 7.4, atmosphere 5% CO<sub>2</sub> in air, temperature 37 °C. For the experiments, fibroblasts were cultured for 3 days in complete growth medium (10% FBS) and then incubated for 7 days with DMEM supplemented with 0.1% FBS, so as to avoid activation of MAPKs by serum growth factors. Medium was renewed on the 3rd day. The osmolality of this medium, determined with a Wescor vapor pressure osmometer, was 310 ± 12 mosmol/kg.

For amino acid-free incubation cell monolayers were washed twice in Earle's Balanced Salt Solution (EBSS) and incubated in the same solution supplemented with 0.1% dialyzed FBS for the indicated periods. The osmolality of EBSS, checked routinely, was 280 ± 13 mosmol/kg ( $n = 6$ ). In several experiments EBSS was supplemented with L-proline, in order to repress the adaptive regulation of system A (8). In other experiments PD98059, an inhibitor of ERK1/2 activation (30), was used; in this case, the drug was added to the medium 90 min before the beginning of amino acid-free incubation and maintained throughout the experiment.

For hypertonic treatment, cells were incubated in a modified DMEM whose osmolality was taken at 400 mosmol/kg with proper additions of sucrose.

**Cell Volume, Transport Activity of System A, and Protein Synthesis—**For these experiments cells were seeded in 2-cm<sup>2</sup> wells of 24-well trays (Falcon) at a density of  $3 \times 10^4$  cells/cm<sup>2</sup>. The cluster tray method (31) has been employed with proper modifications.

Cell volume was estimated from the distribution space of [<sup>14</sup>C]urea (0.5 mM, 2 μCi/ml) determined in 10-min pulses by adding the probe to the extracellular medium, according to a method previously validated in cultured human fibroblasts (17). The experiments were stopped with two rapid washes in 3 ml of ice-cold 300 mM unlabeled urea in water. Ethanol extracts of cells were added to 0.6 ml of scintillation fluid (Hisafe, Wallac) and counted for radioactivity in a Wallac Microbeta Trilux counter. Cell monolayers were then dissolved with 0.5% sodium deoxycholate in 1 N NaOH, and protein content was determined using a modified Lowry procedure (31).

The activity of transport system A was evaluated by measuring the initial velocity of entry of L-[<sup>3</sup>H]proline (0.1 mM, 2 μCi/ml), a site A-specific substrate in cultured human fibroblasts (1). After the indicated periods, cell monolayers were incubated for 5 min at 37 °C in 0.2 ml of EBSS containing the labeled amino acid. The incubation was terminated with two rapid washes with 3 ml of ice-cold 300 mM urea. Cells were extracted with absolute ethanol and extracts counted for radioactivity. Protein contents were determined as described above. In the experiments in which cells were preincubated in EBSS + L-proline, cells were incubated for 90 min in EBSS + 0.1% FBS + 18 μM cycloheximide before the measurement of transport activity. This treatment causes the depletion of intracellular amino acid pool, thus abolishing the trans-inhibition of system A transport activity by the intracellular substrates (1, 8).

Protein synthesis was measured through the determination of L-[<sup>3</sup>H]leucine incorporation in the acid-insoluble fraction. The labeled precursor (5 μCi/ml, 0.1 mM) was added to the medium for 1 h. Cells were then washed three times with 5% trichloroacetic acid and solubilized with 200 μl of 1 N NaOH containing 5% sodium deoxycholate. The radioactivity in trichloroacetic acid-insoluble material was measured by liquid scintillation counting.

**Intracellular Amino Acid Pool—**Cell monolayers were washed twice

with ice-cold phosphate-buffered saline and extracted in a 5% solution of acetic acid in ethanol. The intracellular content of the single amino acid species was determined by high performance liquid chromatography analysis with a Biochrom 20 Amino Acid Analyzer (Amersham Pharmacia Biotech) employing a High Resolution Column Bio 20 Peak Lithium and the Physiological Fluid Chemical Kit (Amersham Pharmacia Biotech) for elution. The column effluent was mixed with ninhydrin reagent, passed through the high temperature reaction coil, and read by the photometer unit. Cell contents of the single amino acid species are expressed as nanomoles/mg of protein.

**Cell Lysis—**Cells, grown in 175-cm<sup>2</sup> flasks, were washed twice with ice-cold phosphate-buffered saline, scraped in the same solution, and collected by low speed centrifugation. The pellet was suspended in 0.3 ml of lysis buffer containing 20 mM Tris-HCl (pH 7.4), 140 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM β-glycerol phosphate, 0.2 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 20 μg/ml leupeptin. Cell lysate, obtained by brief sonication (30 s) in a ice-cold bath, was centrifuged at 15,000 × *g* for 20 min at 4 °C. Protein concentration of the supernatant was determined with the Bio-Rad protein assay, with bovine serum albumin as a standard. Sample aliquots of the supernatant, containing 10–100 μg of proteins, were employed immediately or stored lyophilized or frozen at –80 °C.

**Immunoprecipitation with Anti-phosphotyrosine Antibody—**Aliquots of cell lysates (100 μg of proteins) were incubated for 3 h at 4 °C with monoclonal anti-phosphotyrosine antibody (Santa Cruz Biotechnology) diluted 1:100 in 1 ml of lysis buffer. Samples were then incubated overnight at 4 °C with Protein A/G Plus-Agarose beads (Santa Cruz Biotechnology). The immune complexes, washed four times in lysis buffer and diluted in SDS-PAGE sample buffer, were employed immediately.

**Electrophoresis and Immunoblot—**Protein samples were suspended in SDS-PAGE sample buffer, boiled for 3 min at 95 °C, separated on 10% acrylamide gels by SDS-PAGE, and electrophoretically transferred to a polyvinylidene difluoride membrane. Membranes were blocked in Tris-buffered saline containing 1% casein, 0.33% gelatin, and 1% bovine serum albumin for 2 h at 30 °C. Polyclonal antibodies specific for ERK1, ERK2, JNK1, and p38 were diluted 1:100 in the same solution and added for 1 h at 30 °C. Preliminary experiments had demonstrated that the binding of each antibody was suppressed by 10 μg/ml amounts of the respective MAPK-specific peptide (see Fig. 1C). After four washes with Tris-buffered saline + 0.1% Tween 20, membranes were incubated with horseradish peroxidase-coupled anti-rabbit IgG antibody (Amersham Pharmacia Biotech) at a dilution of 1:1000 at 30 °C for 1 h. The blots were washed extensively and developed using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

**ERKs and p38 Activity Assays—**The overall activity of ERK1 and 2 (ERK1/2 activity) was measured with the Biotrak™ MAP kinase assay (Amersham Pharmacia Biotech). Aliquots of 15 μl of cell lysates, freshly obtained or stored at –80 °C, corresponding to 10 μg of proteins, were incubated at 30 °C with 15 μl of a reaction mixture containing a synthetic specific peptide substrate, related to epidermal growth factor receptor, and Mg-[γ-<sup>32</sup>P]ATP (1 μCi; 1.2 mM). After 30 min the reaction was stopped and samples were spotted on 3-cm discs of binding paper. After several washes in 0.75% phosphoric acid and water, the discs were placed in vials with 10 ml of Hisafe scintillation fluid and counted for radioactivity in a Packard Tri-Carb. Suitable blanks, carried out for the endogenous protein phosphorylation and for nonspecific binding of [γ-<sup>32</sup>P]ATP, were subtracted to activity values.

The activity of p38 was measured with an assay kit from Upstate Biotechnology, based on the determination of MAPKAP kinase-2 activity, which is dependent on its phosphorylation by p38. Equal volumes (10 μl) of cell lysates were incubated at 30 °C in assay buffer (60 μl) containing glutathione *S*-transferase-MAPKAP kinase-2, its substrate peptide (related to human glycogen synthase amino acids 1–9) and Mg-[γ-<sup>32</sup>P]ATP (10 μCi; 0.5 mM). After 30 min, aliquots of 40 μl were transferred on P-81 paper squares, washed five times with 0.75% phosphoric acid and once with acetone. Radioactivity determination and background control were carried out as described for ERKs assay.

**Immunoprecipitation and Activity Assay of JNK—**JNK activity was assayed on immunoprecipitated samples with an assay kit from Upstate Biotechnology. Cell lysates were incubated for 2 h at 4 °C with 1 μg of anti-JNK1 Ab (Santa Cruz Biotechnology). Samples were then incubated overnight at 4 °C with Protein A/G Plus-Agarose beads. The immune complexes, washed three times with lysis buffer and once with kinase assay buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM dithiothreitol), were incubated for 30 min at 30 °C in 30 μl of the same buffer containing 1 μg of c-Jun-(1–169)-glutathione *S*-transferase and Mg-[γ-<sup>32</sup>P]ATP (5 μCi, 0.5 mM). The

TABLE I

Amino acid content of cultured human fibroblasts: effect of amino acid-free incubation

Intracellular content of the indicated amino acids has been measured at various times of incubation in EBSS + 0.1% FBS as described under "Experimental Procedures." The experiment has been repeated twice with comparable results. The cell content of other amino acids not listed was <2 nmol/mg of proteins.

| Amino acid   | Time (min)          |              |              |              |              |
|--------------|---------------------|--------------|--------------|--------------|--------------|
|              | 0                   | 15           | 90           | 180          | 360          |
|              | nmol/mg of proteins |              |              |              |              |
| Asp          | 38.0                | 47.3         | 37.3         | 35.1         | 15.9         |
| Thr          | 83.6                | 67.2         | 32.7         | 24.4         | 9.3          |
| Ser          | 39.9                | 27.7         | 13.4         | 8.8          | 10.5         |
| Glu          | 133.3               | 127.7        | 134.2        | 127.4        | 104.8        |
| Gln          | 230.9               | 168.4        | 75.6         | 65.8         | 16.3         |
| Pro          | 3.1                 | 0.0          | 0.0          | 0.0          | 0.0          |
| Gly          | 52.8                | 45.9         | 33.8         | 30.3         | 31.4         |
| Ala          | 4.3                 | 5.8          | 4.7          | 7.9          | 6.8          |
| Val          | 17.2                | 7.3          | 3.3          | 1.0          | 3.8          |
| Met          | 13.2                | 6.8          | 0.5          | 0.5          | 0.4          |
| Ile          | 17.0                | 4.1          | 3.5          | 3.1          | 2.4          |
| Leu          | 21.0                | 3.6          | 3.0          | 2.5          | 2.7          |
| Tyr          | 13.8                | 9.1          | 2.3          | 1.0          | 2.1          |
| Phe          | 11.2                | 4.0          | 3.9          | 2.5          | 1.6          |
| Lys          | 12.8                | 4.2          | 4.0          | 3.2          | 2.5          |
| Hys          | 6.2                 | 4.3          | 2.1          | 2.7          | 3.2          |
| <b>Total</b> | <b>698.3</b>        | <b>533.4</b> | <b>354.3</b> | <b>316.2</b> | <b>213.7</b> |

reaction was terminated by dilution with an equal volume of 2× SDS sample buffer. After boiling for 5 min, samples were clarified by centrifugation and analyzed by SDS-PAGE, followed by autoradiography to visualize labeled c-Jun.

**Materials**—FBS and DMEM were purchased from Life Technologies, Inc. [<sup>14</sup>C]-Urea (53 mCi/mmol), L-[2-<sup>3</sup>H]proline (52 Ci/mmol), Mg-[γ-<sup>32</sup>P]ATP (3000 Ci/mmol), and L-[4,5-<sup>3</sup>H]leucine (61 Ci/mmol) were from Amersham Pharmacia Biotech. Affinity-purified rabbit polyclonal antibodies and anti-human ERK1, ERK2, JNK1, JNK2, and p38, were obtained from Santa Cruz Biotechnology. SB203580 and PD98059 were obtained from Alexis Biochemicals. The source of all other chemicals was Sigma.

## RESULTS

**Amino Acid Pool, Cell Volume, and MAPK Phosphorylation in Cultured Human Fibroblasts: Effects of Amino Acid-free Incubation**—The incubation of cultured human fibroblasts in amino acid-free EBSS caused a rapid decrease of the intracellular amino acid pool (Table I). Cell content of amino acids was lowered by 50% after a 90-min incubation and by 70% after 6 h of incubation in EBSS. The behavior of the different amino acid species was not homogeneous; glutamine, the most abundant amino acid under control conditions, and the other neutral amino acids were rapidly lost, whereas glutamate, the second most abundant species, and aspartate decreased more modestly after 6 h.

During amino acid starvation the cell volume of cultured human fibroblasts also decreased significantly (Fig. 1A). After 90 min of amino acid-free incubation, cell volume was lower than control by 20%; cell shrinkage reached 30% of the initial value after a 6-h amino acid-free incubation.

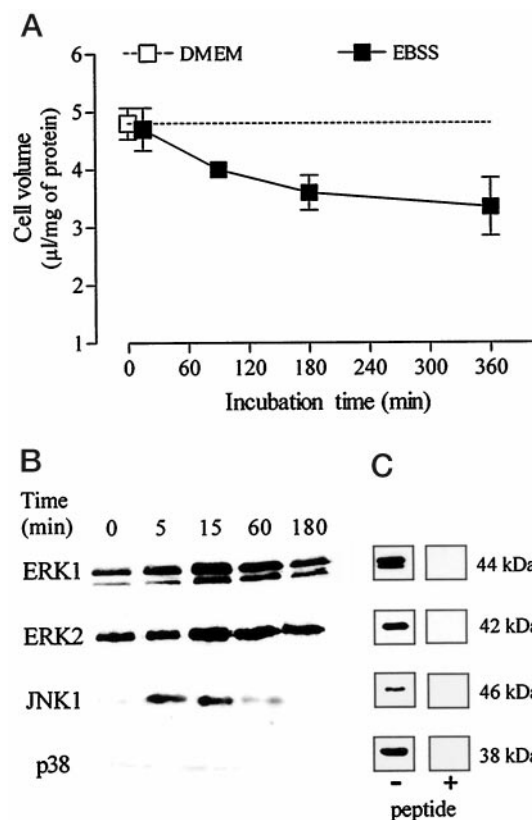
The incubation of human fibroblasts in amino acid-free saline solution caused a selective increase in tyrosine-phosphorylated MAPKs. Results shown in Fig. 1B indicate that Tyr-phosphorylated ERK1, ERK2, and JNK1 evidently increased after 5 min; the maximal increase was observed after 15 min. Thereafter, the amount of phosphorylated ERKs slowly decreased while pJNK1 fell rapidly and disappeared after 3 h of incubation. Apparently, amino acid deprivation did not trigger phosphorylation of p38 at any time of amino acid deprivation. This result cannot be explained by the absence of expression of this MAPK in human fibroblasts. Indeed, preliminary experi-

ments showed that human fibroblasts exhibited clear-cut immunoreactivities not only for the two ERKs and JNK1 but also for p38 (see Fig. 1C); however, no evidence of immunoreactivity was obtained for JNK2 (data not shown).

Lack of p38 phosphorylation in EBSS-incubated human fibroblasts was also demonstrated by the absence of immunoreactivity with monoclonal anti-phospho-p38 antibody (data not shown). Hence, it is concluded that no change in p38 phosphorylation status is detectable upon amino acid deprivation.

**Activity of MAPKs during Amino Acid Starvation**—MAPK activities were measured in cell lysates obtained following different intervals of amino acid deprivation (Fig. 2). A 4-fold stimulation of ERK1/2 activity was observed after 5 min of amino acid-free incubation and maintained for 1 h (Fig. 2, upper panel); compared with control, values remained significantly higher after 3 h of amino acid deprivation.

Fig. 2 (middle panel) indicates that JNK activity exhibited a slight, transient increase after 5 and 15 min of incubation in amino acid-free saline solution. After 1 h JNK activity returned to control values that were maintained thereafter (see also the blot of phosphorylated c-Jun, the product of JNK1 activation, shown in the inset). Amino acid deprivation did not signifi-



**FIG. 1. Cell volume changes and MAPK phosphorylation during amino acid starvation of cultured human fibroblasts.** Panel A, cell volume was measured following the indicated time intervals of amino acid deprivation. Data are means of four independent determinations with S.D. shown when greater than the size of the point. The experiment was repeated twice with comparable results. Panel B, cells were lysed after the indicated times of amino acid-free incubation. Aliquots of lysates (100 μg of proteins) were immunoprecipitated with monoclonal anti-phosphotyrosine antibody. The immunoprecipitates were separated on SDS-PAGE and probed with specific anti-MAPK antibodies. See "Experimental Procedures" for details. Blots from a representative experiment are grouped in the figure; a replicate experiment yielded comparable results. Panel C, samples of cell lysates blotted on polyvinylidene difluoride membrane were probed with specific antibodies for MAPKs in the absence (-) or in the presence (+) of the respective immunizing peptide (10 μg/ml) to demonstrate the specificity of the immunoreactivity.

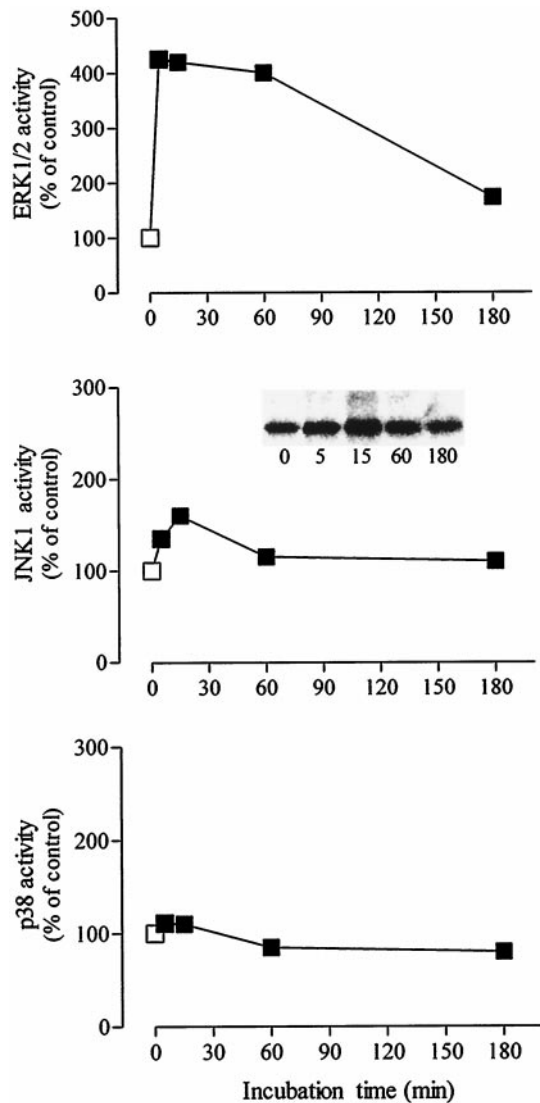


FIG. 2. Effect of amino acid-free incubation on the activities of MAPKs. The activities of ERK1/2 (upper panel), JNK-1 (middle panel), and p38 (lower panel) were measured in cell lysates, obtained after the indicated times of amino acid deprivation (■). For ERK1/2 and p38 activity, values represent  $\gamma$ - $^{32}\text{P}$  radioactivity incorporated into specific substrates. Data of JNK activity represent the densitometric quantification of phosphorylated specific substrate c-Jun shown in the inset. See "Experimental Procedures" for details. All the MAPK data are expressed as a percentage of control values obtained in cells maintained in complete medium (□). Each MAPK activity was determined twice with comparable results.

cantly affect the activity of p38 (Fig. 2, lower panel). These results indicate that ERK1/2 and, to a lesser extent, JNK1 were activated by EBSS incubation, while p38 activity was not affected by this experimental condition.

**Effect of PD 98059 and of L-Proline on EBSS-stimulated Activation of ERK**—To evaluate the relevance of the ERK pathway in adaptive regulation, we suppressed kinase activation with the inhibitor PD98059. This flavone compound binds the inactive form of mitogen-activated protein kinase kinase 1/2, thus preventing ERK1/2 activation (30). As expected, no substantial inhibition of ERK basal activity was produced by the inhibitor. However, no increase was detected in ERK activity upon the incubation of cultured human fibroblasts in amino acid-free saline solution if the cells were pretreated with 50  $\mu\text{M}$  PD98059 for 90 min (Fig. 3, upper panel).

The presence of 1 mM L-proline or of 1 mM MeAIB, a non-metabolizable amino acid analogue, produced characteristic

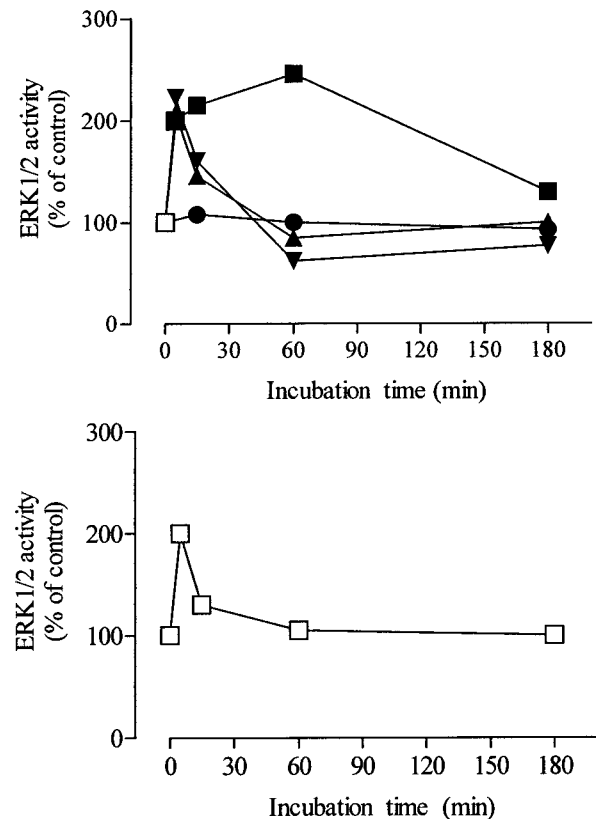


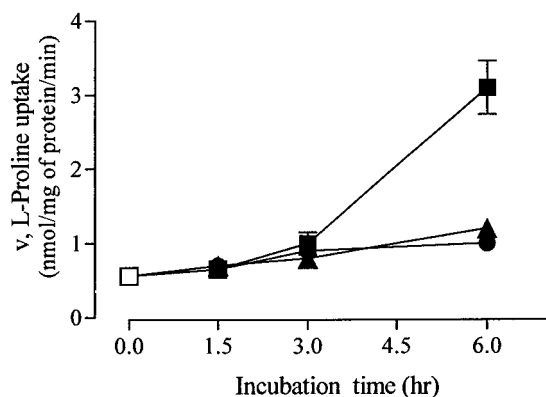
FIG. 3. Effects of PD98059 and amino acids on the activity of ERK1/2. Upper panel, the activity of ERK1/2 (see "Experimental Procedures" for details) was measured following incubation in amino acid-free saline solution (■), or in the same solution supplemented with 1 mM L-proline (▲), 1 mM MeAIB (▼), or 50  $\mu\text{M}$  PD98059 (●). PD98059 was added 90 min before medium substitution. Results are means of two independent determinations expressed as percentage of the control values obtained in cells maintained in complete medium (□). Lower panel, at time 0 the incubation medium was removed and immediately put again in the same dish. The activity of ERK1/2 was measured at the indicated times following the mock substitution of incubation medium. Results are means of two independent determinations expressed as percentage of the control value obtained at time 0 before medium substitution. The experiments shown in both panels were repeated twice with comparable results.

changes in the pattern of ERK activation during amino acid deprivation (Fig. 3, upper panel). Indeed, while the kinase was rapidly activated even in the presence of either amino acid, the activation was transient and rapidly faded after a few minutes. As a result, after 60 min of incubation in EBSS + 1 mM L-proline or in EBSS + 1 mM MeAIB, values of ERK activity were no more significantly different from that exhibited by control cells maintained in complete, amino acid-rich medium.

The pattern of ERK activation observed in the presence of L-proline or MeAIB is comparable to that observed upon mock substitution of extracellular medium with the same medium (Fig. 3, lower panel).

**Adaptive Up-regulation of System A: Effects of ERK Inhibition and Proline Supplementation**—The activity of transport system A, assessed as the initial influx of the transport-specific substrate L-proline, was measured in human fibroblasts at different times of incubation in EBSS (Fig. 4). As expected, amino acid deprivation markedly enhanced the activity of system A. After 6 h of incubation in EBSS, L-proline uptake was 6-fold higher than in control medium.

If PD98059 was added to the medium 90 min before the incubation in EBSS and maintained thereafter, the increase of activity of system A was completely suppressed. Under these conditions PD98059 did not affect protein synthesis, estimated



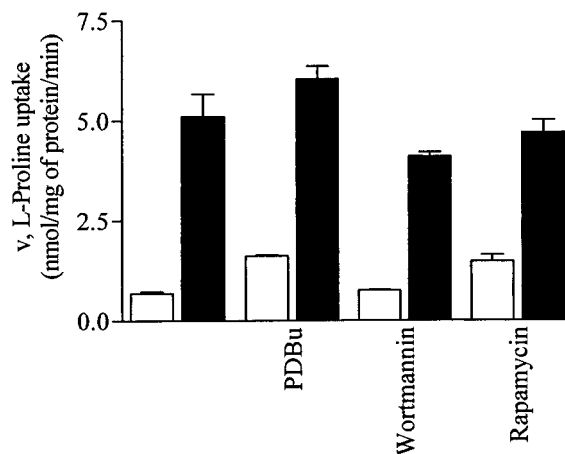
**FIG. 4. Adaptive increase of system A transport activity: effect of L-proline and PD98059.** Cultured human fibroblasts were transferred from complete DMEM ( $\square$ ) into amino acid-free EBSS ( $\blacksquare$ ) or into the same medium supplemented with 1 mM L-proline ( $\blacktriangle$ ) or 50  $\mu$ M PD98059 ( $\bullet$ ). At the indicated times, cells were washed twice in EBSS and the transport activity of system A was assayed as the initial influx of 0.1 mM L-proline in EBSS (see "Experimental Procedures" for details). PD98059 was added 90 min before incubation in EBSS. Data are means of four independent determinations with S.D. shown when greater than the size of the point.

as [ $^3$ H]leucine incorporation in the acid-insoluble cell fraction (data not shown). As expected by previous studies (8), the presence of either L-proline (Fig. 4) or MeAIB (data not shown) completely prevented the increase of transport activity of system A.

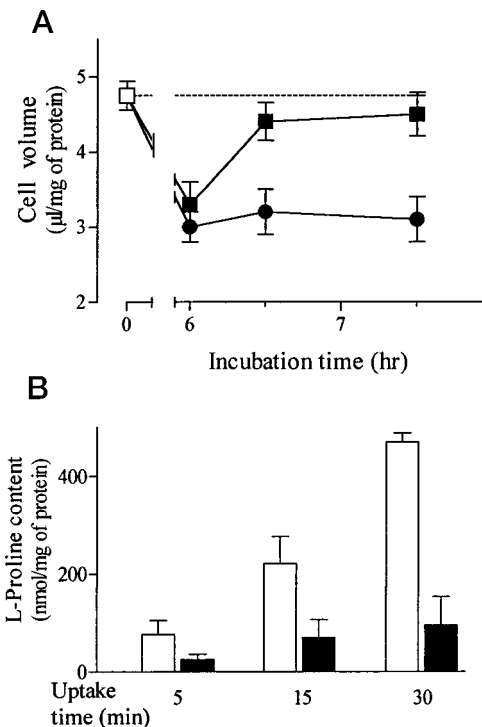
The effect of ERK1/2 pathway inhibition on the adaptive regulation of system A activity appeared to be specific (Fig. 5). No substantial impairment of adaptive regulation was indeed observed after the inhibition of either phosphatidylinositol 3-kinase, obtained with 100 nM wortmannin, or p70S6 kinase, obtained with 100 nM rapamycin. Moreover, chronic exposure to 100 nM PDBu, a treatment that completely down-regulates protein kinase C activity (32), did not hamper the adaptive increase of system A activity.

**Amino Acid-dependent Volume Restoration Is Prevented by PD98059**—As shown in Fig. 1A, cultured human fibroblasts shrank markedly when incubated under amino acid-free conditions; in these cells no effective volume restoration was observed even after an incubation prolonged to 12 h (data not shown). However, if L-proline (1 mM) was added to amino acid-starved, shrunken fibroblasts, cell volume was rapidly restored to values comparable to control cells maintained in complete, amino acid-rich medium (Fig. 6, panel A). This regulatory volume increase was associated to an accumulation of the neutral amino acid into the cells (Fig. 6, panel B); the amount of amino acid taken up by the cells appeared sufficient to account for the volume recovery. On the contrary, if proline was added to amino acid-starved cells in which the adaptive increase of system A transport activity had been suppressed with PD98059 (*cf.* Fig. 4), both volume restoration (panel A) and proline accumulation (panel B) were prevented.

**Hypertonic Treatment of Cultured Human Fibroblasts**—The results presented in Fig. 7 demonstrate that ERK activity undergoes a sustained activation upon incubation of cultured human fibroblasts under hypertonic conditions in complete, amino acid-rich medium. The kinase activity, still significantly higher than control after 60 min of hypertonic incubation, returns to basal values after 3 h. As expected by previous results of our group (17), system A transport activity is markedly enhanced at late times of hypertonic incubation. Transport stimulation is paralleled by the recovery of cell volume.



**FIG. 5. Adaptive increase of system A transport activity: sensitivity to inhibitors of transduction pathways.** Cultured human fibroblasts, maintained in complete medium, have been incubated in fresh complete medium (*open bars*) or in amino acid-free saline solution (*solid bars*) in the absence or in the presence of wortmannin (100 nM), rapamycin (100 nM), or PDBu (100 nM). For PDBu treatment, cells were preincubated with the phorbol ester for 24 h. Data are means of four independent determinations with S.D.



**FIG. 6. Regulatory volume increase of amino acid-starved cultured human fibroblasts.** Panel A, cultured human fibroblasts were transferred from complete medium ( $\square$ ) to amino acid-free EBSS in the absence ( $\blacksquare$ ) or in the presence ( $\bullet$ ) of 50  $\mu$ M PD98059. The inhibitor was added 90 min before amino acids were removed. After 6 h EBSS was supplemented with 1 mM L-proline. Cell volume was determined as described under "Experimental Procedures." The dotted line indicates the volume of cells maintained in complete medium. Panel B, proline accumulation has been determined in parallel cultures during the first 30 min of volume recovery (6–6.5 h) following proline supplementation (*open bars*, without PD98059; *solid bars*, with the inhibitor PD98059). Data are means of four independent determinations with S.D. shown when greater than the size of the point.

## DISCUSSION

The results presented in this study indicate that ERK1/2 are indispensable components of the transduction pathway involved in the triggering of adaptive regulation, the increase in

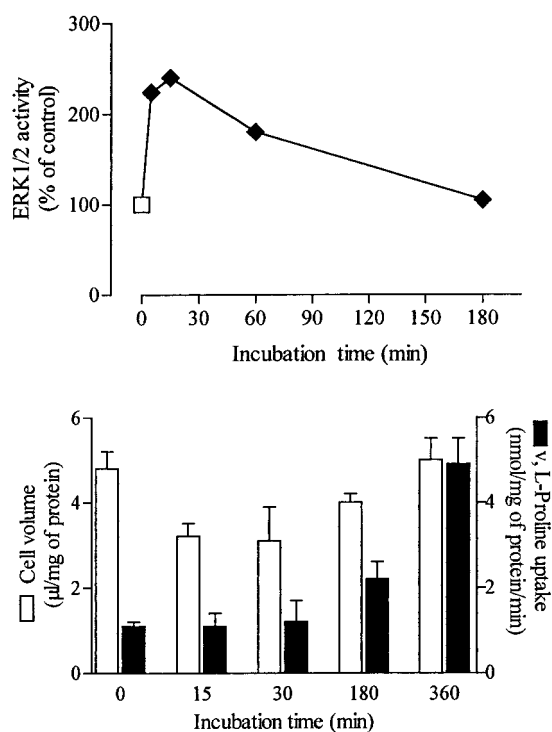


FIG. 7. Effects of hypertonic incubation on the activity of ERK1/2, cell volume, and proline transport in cultured human fibroblasts. Upper panel, the activity of ERK1/2 was measured following incubation in a modified DMEM at 400 mosmol/kg (◆). Results are means of two independent determinations expressed as percentage of the control values obtained in cells maintained in isotonic medium (□). The experiment was repeated twice with comparable results. Lower panel, cell volume (open bars) and proline uptake (solid bars) were measured at different times of hypertonic incubation. For details, see "Experimental Procedures" and the legend to Fig. 4. Data are means of four independent determinations with S.D. shown when greater than the size of the point.

the transport activity of system A observed upon amino acid starvation. This conclusion is based on several lines of evidence. 1) ERK1/2 are markedly, rapidly, and persistently activated when cells are incubated in amino acid-free incubation saline solution, as demonstrated by immunoblots of tyrosine phosphorylated kinases as well as by specific measurement of kinase activity. 2) PD98059, a specific inhibitor of ERK1/2 activation, abolishes the adaptive increase of transport system A without hindering its basal activity. This experimental approach has been widely employed to demonstrate (33) or to exclude (33–36) the involvement of ERK1/2 activation in cell responses to various stresses. 3) When amino acid-free medium is supplemented with a single system A substrate, adaptive increase is prevented (Refs. 5 and 8; this contribution) and only a transient ERK1/2 kinase activation is observed. A mock substitution of extracellular medium with the same medium also produces a transient, although significant, activation of ERK1/2. Thus, what appears to be relevant for the triggering of adaptive regulation is not the activation of ERK1/2 *per se* but, rather, its duration. Additionally, in other models, a sustained activation of ERK is critical for an effective cell response (37).

The fact that ERK1/2 activation is involved in system A adaptive regulation does not imply that it constitutes the sole transduction pathway modified during amino acid deprivation. Indeed, a rapid, transient stimulation of JNK1 was also observed, although the lack of specific inhibitors of this kinase prevents a clear discrimination of its role in the response. Moreover, amino acid deprivation markedly inhibits p70S6 kinase (38–40). In our cells rapamycin increased transport

activity significantly when added to complete medium (Fig. 5), thus suggesting that p70S6 kinase activity is inversely related to transport activity of system A. However, since amino acid-free incubation increases system A activity even in rapamycin-treated cells, p70S6 kinase inhibition does not appear to trigger the adaptive increase. A significant contribution of protein kinase C activation to the adaptive increase of system A activity was also excluded on the basis of evidence obtained with long term PDBu treatment.

ERK1/2-dependent stimulation of system A transport activity appears to be restricted to adaptive regulation and to hypertonic stimulation of the system (see below). Other regulatory mechanisms that lead to stimulation of system A likely employ different transduction pathways. For instance, insulin, which activates the MAPK pathway (41), stimulates system A through a slow mechanism dependent upon protein synthesis (5, 42). ERK activation, however, is not required for the hormone-induced transport stimulation (35). Moreover, insulin effect on system A is blocked by wortmannin that is unable to interfere with the adaptive increase of transport (Fig. 5). The stimulatory changes in system A activity induced by chemical and hyperthermic stress also appear to be ERK-independent (36).

Although p38 is expressed in human fibroblasts, its activation plays no significant role in the adaptive response of system A to amino acid starvation, since there was no detectable change in either the activity or the phosphorylation status of the kinase. A p38-dependent pathway was recently implicated in the hypertonic induction of betaine and myoinositol transport in human monocytes (43) and of betaine transport in Madin-Darby canine kidney cells (44). However, although cell volume changes were not monitored in either of those reports, cell transfer to hypertonic medium is expected to cause an abrupt shrinkage rather than a gradual and progressive decrease of cell volume, as observed here. Moreover, different culture conditions and, in particular, the prolonged maintenance of proliferative quiescence adopted here (7 days in DMEM supplemented with 0.1% FBS) could change the relative sensitivity of the various MAPK pathways, preventing p38 activation. The p38 inhibitor SB203580 (1  $\mu$ M), added to the extracellular medium 60 min before amino acid-free incubation and maintained thereafter, produced a 60% inhibition of adaptive increase in system A transport activity.<sup>2</sup> However, the interpretation of this result is made difficult by the marked activation of JNK1 promoted by the inhibitor in our cells<sup>2</sup> as in other models (44).

ERK1/2 kinases are markedly activated when cultured human fibroblasts (this report) or other cells (20–22) are incubated under hypertonic conditions. In an attempt to identify the signal for ERK1/2 activation and adaptive increase in amino acid-starved cells, we have therefore ascertained whether amino acid-free incubation causes volume changes comparable to those observed under hypertonic conditions. The results obtained demonstrate that incubation under amino acid-free conditions leads to a 30% cell shrinkage that is not reversed, even after prolonged incubation, unless extracellular medium is supplemented with amino acids. The volume loss induced by amino acid starvation is comparable to that observed in cultured human fibroblasts incubated at 400 mosmol/kg (this report), where a vigorous stimulation of system A transport activity is elicited. It should be noted that previous results from our group demonstrated that, also in that case, no volume recovery is possible if substrates of system A are absent (17). These data would suggest that prolonged,

<sup>2</sup> R. Franchi-Gazzola and R. Visigalli, unpublished observations.

uncompensated shrinkage is a condition underlying both hypertonic and adaptive increases of system A activity. On the other hand, as discussed above, if a substrate of system A is present, ERK activation is only transient (Fig. 3) and the adaptive increase is suppressed (Fig. 4). Remarkably, this result is observed with either the natural substrate L-proline or the non-metabolizable analogue MeAIB, thus pointing to an osmotic, rather than to a metabolic, effect of amino acid supplementation.

The relationships between adaptive regulation and hypertonic stimulation of system A are intriguing. Both regulatory mechanisms are dependent upon active protein synthesis (8, 17), involve activation of ERK1/2 (this report), and follow a prolonged cell shrinkage (this report). Moreover, after the development of adaptive regulation, cells are fully competent for a rapid volume restoration if a substrate of system A, e.g. proline, is re-added to the extracellular medium. However, proline-dependent volume recovery is prevented if ERK1/2 activation is inhibited (Fig. 6). These data suggest that the adaptive increase of system A is aimed to allow a rapid recovery of cell volume, once compatible osmolytes substrates of the transport system are provided. More defined conclusions about the macromolecular component(s) synthesized for hypertonic and adaptive stimulation of system A will require molecular data on the system.

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**Adaptive Increase of Amino Acid Transport System A Requires ERK1/2 Activation**  
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