RESEARCH COMMUNICATION

Subcellular localization and adaptive up-regulation of the System A (SAT2) amino acid transporter in skeletal-muscle cells and adipocytes

Russell HYDE, Graham R. CHRISTIE, Gary J. LITHERLAND, Eric HAJDUCH, Peter M. TAYLOR and Harinder S. HUNDAL[1]

Division of Molecular Physiology, School of Life Sciences, Medical Sciences Institute/Wellcome Trust Biocentre Complex, University of Dundee, Dundee DD1 5EH, Scotland, U.K.

The recently cloned amino acid transporter SAT2 is ubiquitously expressed and confers Na^+-dependent transport of short-chain neutral amino acids, characteristics of the functionally defined System A transporter. Here we report the presence of SAT2 mRNA and protein in both skeletal muscle and adipocytes, and the characterization of polyclonal antibodies directed against this transporter. SAT2 protein was present in both plasma-membrane and internal-membrane fractions derived from rat skeletal muscle and adipose tissue, L6 myotubes and 3T3-L1 adipocytes, having a localization similar to that of the glucose transporter GLUT4. Moreover, consistent with the adaptive up-regulation of System A activity following chronic amino acid deprivation, a time-dependent increase in SAT2 protein abundance was observed in amino-acid-deprived L6 myotubes and 3T3-L1 adipocytes. These studies provide the first evidence regarding the subcellular distribution and adaptive up-regulation of SAT2 protein and the characterization of molecular probes for this physiologically important transporter, the function of which is altered in several disease states.

Key words: ATA1, ATA2, membrane transport, methylamino-isobutyric acid (Me-AIB), SAT1.

INTRODUCTION

The plasma membranes of mammalian cells possess multiple transport systems for the cellular uptake and exchange of amino acids [1,2]. Of the classical amino-acid-transport systems that have been functionally described, the best studied has been System A, which mediates the sodium-coupled uptake of short-chain neutral amino acids (e.g. alanine). System A is also capable of transporting amino acids with *N*-methyl substitutions (e.g. *N*-methylaminoisobutyric acid, Me-AIB), a property that has allowed it to be discriminated from other amino acid transporters. A neuronal Na^+-dependent glutamine transporter (GlnT) was recently cloned and, based on its functional characteristics, was proposed as a member of the System A family of transporters [3]. However, the expression of GlnT (now renamed SAT1) is restricted to brain, and, given that functional studies have shown that System A activity is expressed ubiquitously, it seemed highly likely that additional members of this family exist. Indeed, subsequent work from two independent laboratories led to the cloning of a second member of this family, SAT2 (also known as ATA2), which displays a much wider tissue distribution (at least in terms of mRNA expression) and substrate specificity than SAT1 [4,5].

A major feature of System A observed in numerous cell types is its ability to undergo adaptive regulation in response to changes in cellular amino acid availability (reviewed in [1]). Prolonged periods of amino acid deprivation, for example, result in the up-regulation of System A transport activity by a mechanism that is blocked by inhibitors of RNA and protein synthesis [2,6]. This adaptive increase in System A transport does not reflect a generalized cell response, since the activity of other amino acid carriers, such as System ASC, is either unaffected or suppressed during periods of amino acid withdrawal [7]. However, a key issue that has been difficult to resolve is whether the adaptive increase in System A transport is due to increased synthesis of new transporter proteins and/or that of regulatory molecules that stimulate the activity of a pre-existing population of transporters. Here we report, for the first time, that SAT2 protein is expressed in the plasma membrane of skeletal muscle and adipose tissue and that the transporter is also detectable in membranes of intracellular origin. In addition, we provide novel evidence that the increase in functional System A transport induced in response to amino acid deprivation is associated with an increase in SAT2 protein expression in both L6 muscle cells and 3T3-L1 adipocytes.

MATERIALS AND METHODS

Materials

Culture media (α-minimal essential medium, Dulbecco's modified Eagle's medium), foetal-calf serum, and antimycotic/antibiotic solution were obtained from Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). All reagent-grade chemicals were purchased from Sigma–Aldrich Co. Ltd. (Poole, Dorset, U.K.). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG were obtained from the Scottish Antibody Production Unit (Law Hospital, Carluke, Lanarkshire, Scotland, U.K.). Immobilon™P membrane was purchased from Millipore (Bedford, MA, U.S.A.) and reagents for enhanced chemiluminescence were purchased from Pierce and Warriner (Chester, U.K).

Cell culture

L6 muscle cells were cultured to the stage of myotubes in α-minimal essential medium containing 2% (v/v) foetal-calf

Abbreviations used: Me-AIB, *N*-methylaminoisobutyric acid; GlnT, glutamine transporter; HRP, horseradish peroxidase; PM, plasma membrane; LDM, low-density microsomes; HDM, high-density microsomes; IRAP, insulin-regulated aminopeptidase; RT, reverse transcription; IM, internal membrane; MAP kinase, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin.

[1] To whom correspondence should be addressed (e-mail h.s.hundal@dundee.ac.uk).

serum as reported previously [8] and 3T3-L1 cells (provided by Dr Howard Green, Department of Cell Biology, Harvard Medical School, Boston, MA, U.S.A.) were differentiated into adipocytes as described previously [9]. Cells were grown in six-well plates for transport assays or on 15-cm-diameter dishes for isolating cell membranes. Total membranes from 3T3-L1 adipocytes and L6 myotubes were prepared as described previously [8,10]. In some experiments, total membranes from L6 myotubes were subfractionated to isolate plasma membranes and intracellular membranes, as reported previously [8].

Subcellular fractionation of rat skeletal muscle and adipose tissue

Male Sprague–Dawley rats (200 g body weight; Bantin and Kingman, Hull, U.K.) were killed by cervical dislocation prior to excision of hind-limb skeletal muscle and removal of epididymal fat. Skeletal muscle was homogenized and subjected to differential centrifugation for isolation of crude muscle membranes that were subsequently subfractionated on a discontinuous sucrose density gradient (25, 30 and 35 %, w/w) as described previously [11]. This procedure results in the separation of three distinct membrane bands; one on top of the 25 % sucrose cushion (F25), representing membranes enriched with plasma-membrane (PM) markers [11,12], a second band on top of the 30 % sucrose layer (F30), containing intracellular membranes largely of endosomal origin [11], and a third band on top of the 35 % sucrose layer (F35), composed of intracellular membranes endowed with the insulin-sensitive pool of GLUT4 [11,13].

For adipose tissue, adipocytes were isolated from epididymal fat-pads by collagenase digestion using the method of Rodbell [14]. Adipocytes were fractionated to isolate PMs, low-density microsomes (LDM) and high-density microsomes (HDM) as described previously [15]. The protein content of individual adipocyte and muscle fractions was determined using the Bradford method [16].

SDS/PAGE and immunoblotting

Membranes (20 μg of protein) were subjected to SDS/PAGE and immunoblotting as described previously [8]. Separated proteins were transferred on to nitrocellulose membranes and blocked with Tris-buffered saline, pH 7.4, containing 5 % dried skimmed-milk protein and 0.05 % (v/v) Tween-20. Membranes were probed with two polyclonal antibodies (Ab 3340 and Ab 3325) against SAT2 or antibodies against GLUT4 (1F8, 1:1000; purchased from Sigma-GenoSys, Cambridge, U.K.), the insulin regulated aminopeptidase (IRAP, 1:1000; kindly provided by Dr S. Keller, Department of Biochemistry, Dartmouth Medical School, Hanover, NH, U.S.A.) and the α1-subunit of the Na,K-ATPase (Mck-1, 1:100 kindly provided by Dr K. Sweadner, Laboratory of Membrane Biology, Massachusetts General Hospital, Charlestown, MA, U.S.A. [17]). SAT2 antibodies were raised in rabbits against separate regions of the rat SAT2 amino acid sequence using synthetic peptides conjugated to keyhole-limpet (*Diodora aspera*) haemocyanin (Sigma-GenoSys). Ab 3340 was raised against a 14-amino-acid peptide (FLLESNLGK-KKYET) corresponding to an N-terminal region of rat SAT2 and Ab 3325 to a sequence (EELKSRSRRRMMNV) in the extracellular loop between proposed transmembrane domains 7 and 8. Both SAT2 antibodies were used at a final dilution of 1:2500. In some experiments SAT2 antibodies were pre-adsorbed with the respective antigenic peptide (100 μg/ml) overnight at 4 °C and mixed by rotation. Primary antibody detection was performed using either HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG (1:1000). Immunoreactive protein bands were revealed by enhanced chemiluminescence on Konica (Hohenbrunn, Germany) Medical film.

Reverse-transcription (RT)-PCR

System A isoform-specific oligonucleotide primer pairs were synthesized (MWG Biotech AG, Ebersberg, Germany) to match bp 367–390 (SAT1 forward, 5′-CTGATCGGGAGAGTAGGA-GGAGTC-3′) and bp 859–836 (SAT1 reverse, 5′-AGCGGGA-GAATTATGCCAAAGGTT-3′) of the rat neuronal glutamine transporter (SAT1) sequence (AF075704) and bp 595–616 (SAT2 forward, 5′-TACGAACAGTTGGGACATAAGG-3′) and 1293–1272 (SAT2 reverse, 5′-AGTTCCCACGATCGCAGAG-TAG-3′) of the rat SAT2 nucleotide sequence (GenBank® accession number AF173682). Total RNA was isolated from cultured cells (3T3-L1 adipocytes, L6 and C2C12 myotubes) and rat kidney and brain using the RNeasy method (Qiagen). Rat skeletal-muscle total RNA was isolated using TRIzol reagent (Life Technologies) and subsequent protocol. RT-PCR was performed using the Access RT-PCR System (Promega) according to the manufacturer's protocol using the following PCR amplification conditions: 94 °C for 30 s, 55 °C for 1 min, 68 °C for 1 min, repeated for 40 cycles, followed by a single additional extension step at 68 °C for 7 min. RT-PCR products were resolved on a 1.5 % (w/v) agarose/Tris/acetate/EDTA gel (containing ethidium bromide at 0.5 μg/ml) and revealed under UV light.

Measurement of System A amino acid transport

System A activity was assayed by measuring the uptake of Me-AIB as described previously [18]. Briefly, L6 myotubes were incubated with 10 μM [^{14}C]Me-AIB (1 μCi/ml) for 10 min. Non-specific tracer binding was determined by measuring cell-associated radioactivity in the presence of an excess saturating dose of unlabelled Me-AIB (10 mM). Uptake of Me-AIB was determined by aspirating the radioactive medium, followed by three successive washes in ice-cold iso-osmotic saline solution (0.9 % w/v NaCl). Cells were lysed in 0.05 M NaOH, and the associated radioactivity determined by liquid-scintillation counting. Total cell protein was determined by the method of Bradford [16].

In some experiments, cells were amino-acid-deprived for the periods indicated in the Figure legends by incubation in Krebs–Ringer phosphate buffer (KRP; 120 mM NaCl/4.8 mM KCl/1.3 mM $CaCl_2$/1.2 mM $MgSO_4$/3.1 mM NaH_2PO_4/12.5 mM Na_2HPO_4/200 nM adenosine, pH 7.4), containing 5 mM D-glucose and 2 % (w/v) BSA. Control incubations were performed using KRP buffer supplemented with a physiological mix of amino acids of a composition described previously [19]. Following amino acid deprivation, cells were either used for transport assays or for preparation of total cell membranes as described above.

Statistical analysis

Statistical analysis was carried out using a two-tailed Student's *t* test. Data were considered statistically significant at P values $\leqslant 0.05$.

RESULTS AND DISCUSSION

SAT2, but not SAT1, is expressed in skeletal muscle and adipocytes

To determine whether SAT1 and SAT2 mRNA is expressed in skeletal muscle and fat, we performed RT-PCR using rat specific

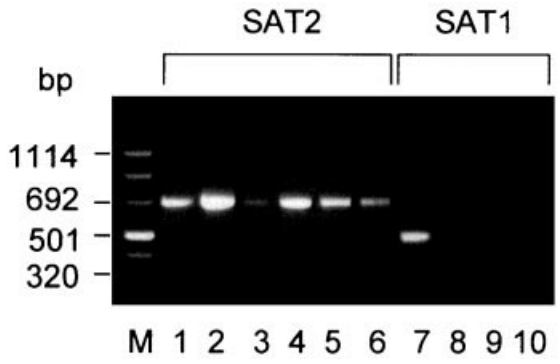

Figure 1 Tissue distribution of SAT1 and SAT2 by RT-PCR

Lanes 1 and 8, rat skeletal muscle; lanes 2 and 9, L6 myotubes; lane 3, C2C12 myotubes; lanes 4 and 10, 3T3-L1 adipocytes; lane 5, rat liver; lanes 6 and 7, rat brain. Lane M, DNA ladder marker VIII (Boehringer-Mannheim). Lanes 1–6, RT-PCR reactions using SAT2-specific oligonucleotide primers; lanes 7–10, reactions using SAT1-specific oligonucleotide primers.

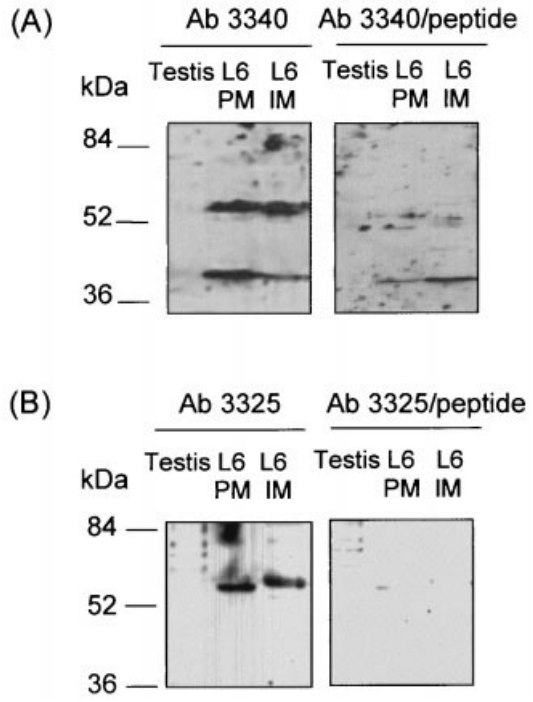

Figure 2 Characterization of SAT2 antibody specificity in L6 myotubes

A 20 μg portion of membrane protein from L6 PM or IM fractions or rat testis were separated by SDS/PAGE. Anti-SAT2 (**A**, 1:2500 Ab 3340; **B**, 1:2500 Ab 3325) antibodies were used to immunoblot SAT2 from L6 myotube fractions, with or without pre-adsorption of the sera with 100 μg/ml antigenic peptide. Blots are representative of three independent experiments.

SAT1 and SAT2 primers. Figure 1 shows that rat skeletal muscle, rat L6 myotubes, mouse C2C12 myotubes, murine 3T3-L1 adipocytes, rat liver and brain generated the expected 693-bp PCR fragment for SAT2. In contrast, SAT1 mRNA was detected only in rat brain. As a negative control, an equal volume of water replaced avian-myeloblastosis-virus reverse transcriptase in the RT-PCR reaction and, under these conditions, no amplified products were obtained (results not shown). These findings are in line with the observations of Yao et al. and Sugawara et al. [4,5], and confirm that SAT2 is more widely distributed than SAT1 and that the latter does not appear to be expressed in muscle cells or adipocytes.

Subcellular distribution of SAT2 in rat skeletal-muscle cells and adipose tissue

In an attempt to gain some insight into the subcellular localization and regulation of SAT2 protein, we raised antibodies against two different epitopes. One of these corresponded to an N-terminal intracellular region of rat SAT2, whereas the other was located in the putative extracellular loop between proposed transmembrane domains 7 and 8. Figure 2 shows that SAT2 antibodies to both the N-terminal (Ab 3340) and extracellular domain (Ab 3325) reacted with a protein of $\approx$ 60 kDa in membranes prepared from L6 muscle cells. On the basis of the proposed amino acid sequence the predicted molecular size for SAT2 is $\approx$ 55 kDa; however, the protein does possess two potential glycosylation sites, and these may alter its mass and mobility in SDS-containing gels. Neither antibody detected this 60 kDa band in membranes prepared from rat testis, consistent with the finding that this tissue does not express any detectable SAT2 mRNA [4]. Furthermore, the protein bands corresponding to SAT2 with either Ab 3340 or Ab 3325 were not detectable when membranes were probed using serum that had been pre-adsorbed with the appropriate antigenic peptide. The 3340 antibody also detected an additional band of $\approx$ 40 kDa that was not observed with Ab 3325 (Figure 2A). The significance of this protein band is unclear at present, but may represent the immature non-glycosylated form of SAT2. An alternative possibility that we cannot exclude is that this band may be a protein that is unrelated to SAT2, but which happens to share the epitope sequence to which the antibody has been raised.

The data in Figure 2 also show that SAT2 was detectable in microsomal or intracellular membranes prepared from L6 cells. This observation is consistent with the possibility that muscle cells sequester this carrier in an intracellular compartment, which may be 'tapped' in response to hormonal and stress stimuli that are known to stimulate acutely the activity of System A [20,21]. Although, the SAT2 signal obtained in the internal membrane (IM) fraction with Ab 3325 appears to migrate with a slightly higher molecular mass than that observed in the PM fraction, this was not a consistent finding (Figure 2B). It is also noteworthy that these same membrane samples when immunoblotted with Ab 3340 did not show any detectable differences in the SAT2 migration between the PM and IM fractions (Figure 2A). To determine whether this intracellular localization could also be observed in rat skeletal muscle and adipose tissue, we subsequently prepared PMs and intracellular membranes from these tissues and screened them with our SAT2 (Ab 3325) antibody. Figure 3(A) shows that a single immunoreactive band of $\approx$ 60 kDa was observed in membrane fractions termed F25, F30 and F35 prepared from skeletal muscle. The strongest SAT2 signal was observed in F25, which is a PM-enriched fraction. The upper panel shows that this fraction is enriched in the α1 subunit of the Na,K-ATPase, a PM marker that is not present to any significant extent in the F30 and F35 fractions [12,22]. In contrast, SAT2 was detectable in membranes of endosomal origin (F30, [11]) and those ostensibly of intracellular nature (F35). Given that the total protein recovery of the F30 and F35 is typically greater than the F25 (by up to fivefold, [11]), the presence of SAT2 in these fractions represents a sizable intracellular pool. Likewise, when PMs, LDM and HDM were prepared from rat adipose tissue, we were able to detect SAT2 protein expression in

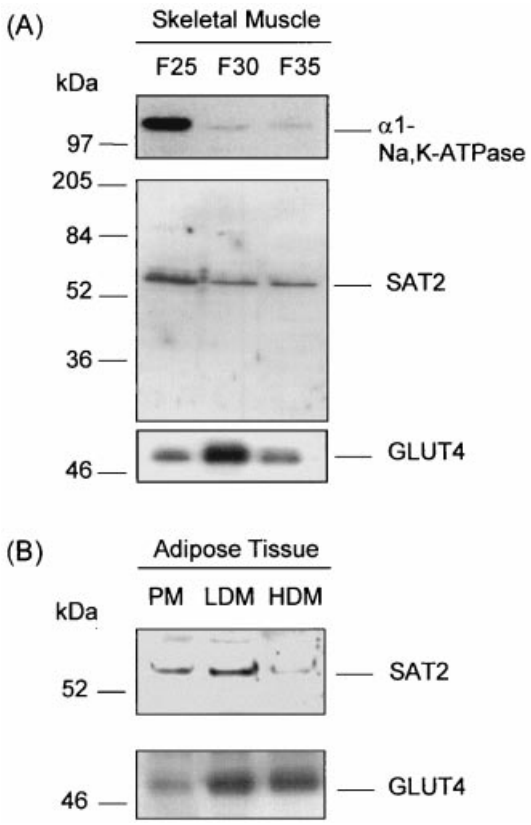

Figure 3 Subcellular distribution of SAT2 in rat skeletal muscle and adipose tissue

A 20 μg portion of protein from subcellular fractions of (**A**) rat hind-limb skeletal muscle (F25, F30 and F35; see the text) and (**B**) rat epididymal fat-pads (PM, LDM and HDM) were separated by SDS/PAGE and subject to immunoblotting with anti-SAT2 serum (1:2500, v/v), 1F8 anti-Glut4 (1:1000, v/v) or anti-(α1-subunit of Na,K-ATPase) (1:100, v/v) antibodies. Blots are representative of three independent experiments.

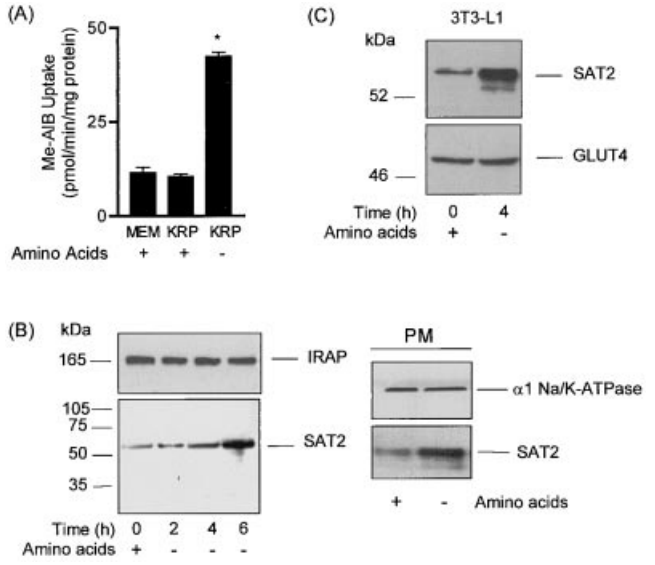

Figure 4 Adaptive upregulation of SAT2 protein following amino acid deprivation

L6 myotubes or 3T3-L1 adipocytes were incubated in KRP buffer for periods up to 6 h in the presence or absence of an amino acid mixture. (**A**) Following a 6 h pre-incubation in KRP alone, KRP plus amino acids, or serum-free αMEM, Me-AIB uptake was assayed in L6 myotubes. Total membranes were isolated from (**B**) L6 myotubes and (**C**) 3T3-L1 adipocytes following amino acid deprivation in KRP for the indicated times. In some experiments (**B**, right), PMs were isolated from L6 myotubes following amino acid deprivation in KRP for 4 h. A 20 μg portion of protein was separated by SDS/PAGE and Western-blotted with anti-SAT2 serum (1:2500, v/v), anti-IRAP (1:1000, v/v), Mck-1 (α1-subunit of Na,K-ATPase, 1:100, v/v) or 1F8 anti-Glut4 antibody (1:1000, v/v). Values in (**A**) are means $\pm$ S.E.M. for six separate observations, whereas immunoblots in (**B**) and (**C**) are representative of two independent membrane-fractionation experiments.

both the PM and the intracellular LDM fraction, with a much weaker SAT2 signal detected in the HDM. The subcellular distribution of GLUT4 in the respective muscle and fat fractions is also shown (Figure 3). The muscle F35 and adipocyte LDM fractions house the insulin-recruitable pool of GLUT4 [11,23], and the finding that both fractions contain SAT2 raises the interesting possibility that SAT2 and GLUT4 may reside in a common membrane compartment.

Adaptive upregulation of System A activity and SAT2 protein expression

One of the best documented aspects of System A regulation is the ability to increase amino acid uptake via this transport system in response to withdrawal of extracellular amino acids (i.e. adaptive up-regulation) [2]. However, it is unknown whether this adaptive increase in System A activity, which relies upon on-going protein synthesis, is due to increased expression of new System A transporters or to changes in the expression of ancillary molecules (e.g. enhancers or repressors) involved in System A regulation. To address this issue we investigated the effects of amino acid deprivation on System A activity and SAT2 expression in L6 myotubes. Figure 4(A) shows that a 6 h period of amino acid deprivation induced a 4-fold increase in System A transport. Under these conditions, total membranes from L6 myotubes (immunoblotted using our SAT2 antibodies) displayed a more-than-3-fold increase in SAT2 protein expression (Figure 4B). The increase in SAT2 expression was time-dependent, as we were able to detect incremental increases in SAT2 abundance over a 6 h amino-acid-deprivation period (Figure 4B). To confirm that the observed increase in System A transport correlated with an increase in cell-surface SAT2 protein, we isolated PMs from L6 cells following a 4 h amino-acid-deprivation period. This treatment protocol resulted in just over a 3-fold increase in SAT2 protein in the PM (Figure 4B). This increase was not observed for the α1-subunit of the Na,K-ATPase, a classical marker protein of the PM, indicating that the increase in SAT2 abundance did not form part of a generalized cell response to amino acid withdrawal. The adaptive increase in SAT2 protein was not restricted to muscle cells, as similar findings were also obtained in 3T3-L1 adipocytes that had been subjected to a 4 h period of amino acid deprivation (Figure 4C). A weak immunoreactive signal corresponding to a protein of $\approx$ 55 kDa was also detectable in total membranes prepared from 3T3-L1 adipocytes following withdrawal of extracellular amino acids (Figure 4C). This band most likely represents the non-glycosylated form of SAT2, which has a predicted molecular size of 55 kDa [4], and its appearance is consistent with the idea that amino acid deprivation induces *de novo* synthesis of SAT2 transporters. It is noteworthy that, unlike SAT2, the expression of IRAP and GLUT4 was unaltered by cellular amino acid deprivation (Figures 4B and 4C). This latter finding indicates that the observed changes in SAT2 expression do not represent a generalized cell response to amino

acid withdrawal and, moreover, could not be attributed to aberrant loading of samples on SDS-containing gels.

The precise mechanisms by which an increase in System A transport activity is instigated in response to amino acid withdrawal remains poorly understood, but the concept that amino acids are potent regulators of gene expression and cell signalling is an area of intense current interest (for recent reviews, see [24,25]). There is some evidence that implicates components of the classical mitogen-activated protein (MAP) kinase pathway in the adaptive regulation of System A in cultured human fibroblasts, based on the finding that the MAP kinase kinase (also known as MEK) inhibitor PD 98059 attenuates significantly the increase in System A activity [26]. In these cells it has been suggested that amino acid deprivation elicits changes in cell volume that subsequently activate the MAP kinase pathway. This pathway is known to stimulate the activity of certain transcription factors [e.g. activator protein-1 ('AP-1')], and it is plausible that one or more of these may bind to putative amino acid response elements in the vicinity of the SAT2 gene promotor, providing a possible mechanism by which System A expression is modulated. Other potential signalling pathways that may regulate SAT2 expression include the mammalian target of rapamycin (mTOR)/p70 S6 kinase pathway which is regulated by amino acid availability [27,28] and which we have shown recently to be involved in the regulation of System A activity in response to changes in leucine availability [29]. mTOR is known to play a key role in regulating the activity of protein components involved in mRNA translation, but precisely how mTOR signalling is modulated by amino acid supply is an issue that is poorly understood at present.

Our results also highlight for the first time that SAT2 protein is localized in membranes that are ostensibly of intracellular origin in both skeletal muscle and adipose tissue. In these tissues, System A transport is also stimulated acutely by insulin [21,30,31], and it is plausible that localization of SAT2 in these intracellular compartments represents a pool of System A carriers that may be rapidly recruited to the PM in response to insulin (e.g. in a manner similar to that reported for the insulin-responsive GLUT4 transporter [32]). In skeletal muscle, a tissue which represents the body's major store of amino acids, this increase in System A transport activity may be important in helping to dispose of circulating amino acids following a meal and ensuring an adequate supply of precursor molecules for protein synthesis. During diabetes, however, System A activity becomes dysfunctional [33], and this may have important consequences for the normal maintenance of muscle protein mass. The availability of SAT2-specific molecular probes will now allow assessment of the expression, subcellular distribution and regulation of SAT2 in response to insulin as well as during conditions of insulin resistance.

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