

mNAT3, an N-system Amino Acid Transporter Expressed in Hepatocytes and Regulated by an Insulin-activated and PI3 Kinase-dependent Signaling

Sumin GU*, Paul LANGLAIS*, Feng LIU†*, and Jean X. JIANG*¹

*Departments of Biochemistry and †Pharmacology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900

Short title: Amino acid transporter and regulation by insulin-PI3 signaling

Key words: amino acid transporter, mNAT3, liver, insulin signaling, PI3 kinase

¹ To whom correspondence should be addressed

Jean X. Jiang, Ph.D.

Department of Biochemistry

University of Texas Health Science Center

7703 Floyd Curl Drive

San Antonio, TX 78229-3900

Tel: (210) 567-3796 Fax (210) 567-6595

e-mail: jiangj@uthscsa.edu

Abstract

Amino acid transporters are essential for normal cell function and physiology. We report here the identification, and functional and regulatory characterization of a mouse amino acid transporter mNAT3. Expression of mNAT3 in *Xenopus* oocytes revealed that the strongest transport activities were preferred for L-alanine. In addition, mNAT3 is a Na⁺ and pH-dependent, low-affinity transporter and partially tolerates substitution of Na⁺ by Li⁺. mNAT3 was found to be predominantly expressed in the liver, where it is localized to the plasma membrane of hepatocytes, with the strongest expression in those cells adjacent to the central vein, decreasing gradually towards the portal tract. Treatment of mouse hepatocyte-like H2.35 cells with insulin led to a significant increase in the expression of mNAT3, and this stimulation was associated closely with an increase in the uptake of L-alanine. Interestingly, this insulin-induced stimulatory effect on mNAT3 expression was attenuated by the PI3 kinase inhibitor LY294002, but not by the MAP kinase inhibitor PD98059, although both kinases were fully activated by insulin. The data suggest that insulin-mediated regulation of mNAT3 is likely to be mediated through a PI3 kinase-dependent signaling pathway. The unique expression pattern and insulin-mediated regulatory properties of mNAT3 suggest that this transporter may play an important role in liver physiology.

INTRODUCTION

Amino acid transporters play essential roles in a variety of cellular processes, including uptake of nutrients, energy and chemical metabolism, detoxification, and specifically the nitrogen metabolism in the liver [1,2]. Mammalian amino acid transporters identified to date belong to a variety of gene families including CAT, A, N, and EAAT [3-5]. Many of the gene families identified thus far consist of multiple members that are expressed in a tissue specific manner. Recently, we identified and characterized three N-system amino acid transporters; mNAT¹ and mNAT2, which are predominantly expressed in mouse liver and retina, and hNAT3 is predominantly expressed in human liver [6-8]. The orthologous genes of mNATs have been identified by our laboratory and others; human g17 and rat SN1 [6,9,10] are orthologs of mNAT; rat GlnT [11] is an ortholog of mNAT2, and rat system A transporter ATA3 [12] is an ortholog of hNAT3 and human ATA3 [13]. Both systems, N and A, mostly convey L-glutamine, L-histidine, and L-alanine across cell membranes. Transport of glutamine and alanine is essential for control of nitrogen metabolism in the liver [2,14]. In the liver, glutamine is involved in the detoxification of ammonia and in the production of urea [15]. Alanine directly participates in the nitrogen metabolism between liver and muscle for glycolysis and in the removal of ammonia from muscle. In addition, we have observed a graded distribution of mNAT expression from the central vein to the portal tract [6].

Previous studies have shown that activities of N and A transport systems are regulated by growth factors and hormones (including insulin in some tissues), substrate availability, cell swelling, starvation, etc. [16-20]. In addition, activities of systems N and A transport are up-regulated in the liver during prolonged fasting and corticosteroid treatment, becoming the rate-determining step in intrahepatic metabolic processes [17]. Although previous studies have shown that activities of system N and A transport appear to be regulated by physiological factors, the precise regulatory mechanism at the molecular level is unknown due to a lack of information regarding the identity of transporter proteins. The recent progress in molecular cloning permits the investigation of function and regulation of these transporters in close detail.

In this study, we report the molecular identification and functional characterization of a mouse N-system transporter, mNAT3. mNAT3 was found to be predominantly expressed in mouse liver hepatocytes, with the strongest expression in those cells adjacent to the central vein. More importantly, the expression and function of mNAT3 were stimulated by insulin. This

stimulatory effect appears to be mediated through the PI3 kinase signaling pathway. Its specific expression in the liver, selective transport properties, and specific regulation by insulin suggest that this transporter plays a key role in liver physiology and function.

MATERIALS AND METHODS

Materials

mMESSAGE mMACHINE used for *in vitro* transcription was obtained from Ambion (Austin, TX). The Marathon cDNA Amplification kit and mRNA Separator kit were purchased from CLONTECH (Palo Alto, CA). TRI REAGENT was obtained from Molecular Research Center (Cincinnati, OH). [³H]-labeled L-alanine, L-glutamine, L-glutamate, L-histidine, and L-lysine were purchased from PerkinElmer Life Science (Boston, MA). Restriction endonucleases were purchased from New England Biolabs (Revere, MA). Protease inhibitor cocktail tablets were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Nitrocellulose membrane was purchased from Schleicher & Schuell (Keene, NH) and Nylon transfer membrane-Hybond H⁺ was from Oncor (Gaithersburg, MD). Protein and RNA standard mixture, Oligo(dT) primer, and Superscript II reverse transcriptase were obtained from Invitrogen (Carlsbad, CA). Micro BCA Protein Assay Reagent kit was from Pierce (Rockford, IL). X-OMAT AR films were from Eastman Kodak (Rochester, NY). Paraformaldehyde (16% stock solution) was from Electron Microscopy Science (Fort Washington, PA). Tissue-Tek OCT compound was from Miles Scientific (Naperville, IL). Antibodies against phospho-p44/42 MAP kinase¹ (Thr²⁰²/Tyr²⁰⁴), p44/42 MAP kinase, phospho-Akt (Thr³⁰⁸), and Akt were from Cell Signaling Technology (Beverly, MA). All other chemicals were from either Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Cloning of mNAT3 cDNA

The RT-PCR¹ approach was utilized to identify additional member(s) of the mouse N-system transporter family. RNAs were isolated from various mouse tissues by TRI REAGENT. To obtain the cDNA sequence, polyadenylated RNA was isolated through a mRNA Separator kit. mRNA was converted to single stranded cDNA by oligo(dT) and Superscript II reverse

transcriptase. Several primer pairs were designed based on the homology between conserved regions of the N system amino acid transporters, mNAT (AF159856), SN1 (AF273025), g17 (U49082), mNAT2 (AF184240), and hNAT3 (AF193836). After PCR amplification over 30 cycles with annealing temperatures of 50°C, one of the primer pairs (sense primer: 5'-ATGGATCCCATGGAAGTGGAG-3'; antisense primer: 5'-CAGTGTCTTCATTAGCAAATTG-3') generated a 0.14 kb¹ DNA fragment from mouse liver cDNA. After sequencing (DNA Sequencing Facility, University of Texas Health Science Center at San Antonio (UTHSCSA)), the fragment showed homology to hNAT3 and its corresponding ortholog. A mouse liver cDNA was prepared using the Marathon-cDNA kit according to the manufacturer's instructions. Based on the sequence obtained from this 0.14 kb DNA fragment, two unique primers were designed: 5'-GGAGTTTCCATGCCGGTGTAGCT-3' to amplify the additional 5' sequence; and 5'-CGCGGGGACAGTATTCAGGACAGC-3' to amplify the additional 3' sequence of this new gene. Subsequently, based on the newly generated additional sequence, six specific primers were used to generate a complete ORF¹ sequence of mNAT3.

Subcloning and preparation of cRNA for microinjection in *Xenopus laevis* oocytes

The cDNA was synthesized by PCR and subcloned into the *Xenopus* expression vector containing the 5'- and 3'-flanking sequences of the *Xenopus b* globin gene as previously described [21]. The primer pairs used to amplify the entire ORF of mNAT3 were designed with restriction sites *Bam* HI at the 5' end and *Hind* III at the 3' end; a sense primer containing a restriction site for *Bam* HI and a start codon: 5'-ATGCGGATCCCAAATGGACCCCATGGAAGTGGAG-3'; an antisense primer including the 3' end coding sequence and a stop codon: 5'-ATCGAAGCTTGATTAGTGGTGGATTGGGATTTCG-3'. PCR products were purified and digested with *Bam* HI and *Hind* III before subcloning into the *Xenopus* expression vector. The constructs were confirmed by sequencing. The plasmids were linearized with *Not* I and cRNA was *in vitro* transcribed by T7 RNA polymerase using mMESSAGE mMACHINE. Capped cRNA was extracted with phenol/chloroform, precipitated with ethanol as described [21], dissolved in DEPC¹ treated H₂O at a concentration of 1.5-2.0 µg/µl, and stored at -80°C prior to use for microinjection into *Xenopus* oocytes.

Antibody preparation

Rabbit anti-mNAT3 IgG antibody was produced using a GST¹ tagged fusion protein as described [22]. A DNA fragment encoding amino acids 1-59, which is distinguished from mNAT, mNAT2, and their corresponding orthologs, was produced by PCR using an mNAT3 DNA clone as a template (sense primer: 5'-GCGGGTATCCATGGACCCCATGGAACTG-3', antisense primer: 5'-GCGGAATTCAACCCATTTGTCAGGAACT-3'). This fragment was inserted into the expression vector, pGEX-2T. The recombinant fusion protein was expressed in *Escherichia coli*, induced by IPTG¹, and isolated with GST-beads. The purified fusion protein was used to raise polyclonal antiserum in a rabbit (Pocono Rabbit Farm and Laboratory Inc, Canadensis, PA). The antiserum generated was affinity purified by passage through two Sepharose CL-4B columns, GST-conjugated and GST-mNAT3 fusion protein conjugated, respectively.

Immunofluorescence staining and confocal laser microscopy

The immunofluorescence detection of mNAT3 was performed as described [23]. Oocytes and mouse liver tissues were fixed in 2% paraformaldehyde for 2 h, incubated in 30% sucrose in PBS at 4°C overnight, embedded in OCT, and then frozen in liquid nitrogen. Frozen sections (10 µm thickness) were fixed in acetone at -20°C for 5 min. H2.35 cells were fixed in 2% paraformaldehyde for 30 min. Fixed sections of oocytes, liver, and cells were incubated with blocking solution (2% normal goat serum, 0.25% Triton-X-100, 2% fish skin gelatin, and 1% BSA in PBS) for 30 min and then were incubated at 4°C overnight with affinity-purified anti-mNAT3 antibody (1:300 dilution in blocking solution). The primary antibodies were labeled for 2 h at room temperature by FITC¹-conjugated goat anti-rabbit Ig (1:500 dilution). The specimens were analyzed using a confocal laser scanning microscope (model: Fluoview, Olympus) with excitation at 488-nm by an argon laser.

Transport assay in *Xenopus* oocytes and H2.35 cells

The transport studies using *Xenopus* oocytes were performed based on our previous work [6,8]. Briefly, *Xenopus laevis* oocytes were injected with 50 nl of synthetic cRNA or DEPC-H₂O as a control and were cultured for 2-3 days at 18°C. Oocytes, in groups of five to ten per assay, were rinsed briefly in uptake buffer (2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES, and 50 mM Tris) in the presence of 100 mM NaCl (Na⁺-buffer). These oocytes were transferred into a 24-well culture dish containing 2 ml uptake buffer and were incubated for 2 min at room

temperature. Amino acid transport activities were measured by incubating oocytes in 0.5 ml of uptake buffer in the presence of 50 μM or 500 μM L-amino acids plus 2 μCi [^3H] labeled L-amino acids as tracers for designated time periods. Prior to uptake experiments, preliminary experiments were conducted with the transport measured at various time points. The uptake rate after 30 min incubation is close to the maximal retention of radiolabeled amino acids inside oocytes than any other time points tested. The oocytes were then washed 4 times in cold uptake buffer and lysed in 200 μl of 2% SDS. The radioactivity accumulated by each oocyte was measured with a scintillation counter (Beckmann) in 10 ml of scintillation solution.

The specificity of mNAT3-mediated amino acid uptake was examined using an amino acid competition assay. The uptake of L-alanine at the concentrations of 50 μM and 1 mM was measured in the presence of 5 mM 20 non-radioactive L-amino acids and MeAIB¹. The Na⁺-dependence and Li⁺-tolerance of L-alanine transport by mNAT3 were investigated using Na⁺-buffer, choline⁺-buffer or Li⁺-buffer. The effects of extracellular pH on L-alanine uptake mediated by mNAT3 were investigated at pH 6.5-8.5 by adjusting the Na⁺-buffer with Tris-base or hydrochloric acid as described [24,25]. All experiments were repeated at least three times and the data collected are presented as SEM.

For analysis of the uptake of L-alanine in H2.35 cells, cells were treated in the presence or absence of 0.5 $\mu\text{g/ml}$ of insulin for 24 h, followed by rinsing three times with uptake buffer (150 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 0.8 mM MgCl₂, 5 mM glucose, 10 mM HEPES, pH 7.4). These cells were incubated in uptake buffer for an additional 10 min, transferred to uptake buffer containing 50 μM [^3H]-labeled L-alanine for 5 min, and then washed three times with cold PBS. The cells were lysed in lysis buffer (2% SDS in PBS) and 200 μl of cell lysates was transferred to scintillation solution and the radioactivity of the cells was measured.

Northern blot analysis

Northern blots were performed as described [22]. Total RNAs were extracted from different tissues of adult mice and H2.35 cells using TRI REAGENT according to the manufacturer's instructions. Thirty micrograms of RNA were loaded, separated by agarose gel electrophoresis containing formaldehyde, and transferred to a Nylon membrane. The membrane was hybridized at 45°C for 12 h in a hybridization solution containing 50% formamide and [^{32}P]-labeled cDNA

probe corresponding to nucleotides 160-330 of mNAT3. The probed membrane was washed in a high-stringency solution of 0.1 X SSC and 0.1% SDS at 63°C for 1 h.

Membrane protein preparation and immunoblot analysis

Crude membrane extracts were prepared from liver, H2.35 cells, and neuroblastoma-like N2A cells as described previously [6,26,27]. The lysates from liver, H2.35, and N2A cells were centrifuged at 100,000 X g at 4°C for 30 min. The membrane pellet was collected and the protein concentration was determined using the Micro BCA Protein Reagent Assay kit. Twenty micrograms of protein were loaded, separated on a 10% SDS-PAGE, and transferred to a nitrocellulose membrane. The membranes were blotted with antibodies against mNAT3 (1:300 dilution) and β -actin (1:5000) for samples from liver, H2.35, and N2A cells, and antibodies against phospho-p44/42 MAP kinase (Thr²⁰²/Tyr²⁰⁴, 1:2000) and phospho-Akt (Thr³⁰⁸, 1:5000) for samples from H2.35 cells. The membranes blotted with antibodies to phospho-MAP kinase and -Akt were extensively washed and reblotted with p44/42 MAP kinase antibody (1:1000) and Akt antibody (1:1000), respectively. The primary antibodies were detected using secondary anti-rabbit antiserum (1:5000) for mNAT3, MAP kinase, Akt, and phospho-Akt or anti-mouse antiserum (1:5000) for β -actin and phospho-MAP kinase. These secondary antibodies were conjugated with either peroxidase or alkaline phosphatase and were detected using either chemiluminescence reagent kit (ECL) or alkaline phosphatase colorimetric reaction, respectively. The membranes treated with ECL were exposed to X-OMAT AR films and detected by fluorography.

Statistic analysis

Data were analyzed using one-way ANOVA and Student-Newman-Keuls multiple comparison test with the InStat biostatistic program (GraphPad software). Data are presented as the mean \pm SEM with more than three determinations. *Asterisks* indicate the degree of significant differences as compared with the controls (** $p < 0.005$).

RESULTS

Molecular Cloning of mNAT3 and Sequence Analysis

We used PCR-homology cloning to identify member(s) of N-system transporters. cDNAs from various mouse tissues were screened using conserved primer pairs. One fragment of 0.14 kb was obtained from liver tissue. This fragment showed 86% and 92% nucleotide identity to hNAT3 nucleotides 188-225 and 299-324, respectively, and 82% to rat ATA3 nucleotides 188-324. Further cloning using primers derived from this PCR fragment sequence generated a complete ORF of the new gene which encodes 547 amino acid residues (Genbank² accession # AB055004) with predicated molecular mass of 60.6 kDa. This new protein shares 55% and 44% amino acid homology with mNAT (AF159856) [6] and mNAT2 (AF184240) [7], respectively. However, the homologies between this protein, and hNAT3 (AF193836) [8,13] and its rat ortholog ATA3 (AF295535) [12] are much higher with 87% and 91%, respectively. The results of sequence analysis suggest that this protein is the mouse ortholog of hNAT3 and ATA3, thereby named as mNAT3. The nucleotide and amino acid sequences of mNAT3 are shown in Fig. 1. The first seven transmembrane domains are highly conserved between mNAT family members while N-terminal region prior to the first transmembrane domain is highly diverse. Based on hydrophobicity analysis (PSORT version 6, Nakai file server; TMPRED, Swiss, EMBNET; BCM Search Launcher), mNAT3, like other members of the mNAT family, is a plasma membrane protein (certainty = 69.6%) with a high probability of having 10 transmembrane domains. As the deduced sequence lacks a signal peptide, the long N- and the short C-terminal tails are predicted to be in the cytoplasm (high probability score).

mNAT3 Preferably Transports L-alanine, L-histidine, and L-glutamine.

To determine the function of mNAT3, we expressed mNAT3 in *Xenopus* oocytes, which is one of the model systems often used to study the properties of transporters [6,28,29]. Using an affinity-purified mNAT3 antibody, expression of mNAT3 was detected by immunofluorescence analysis as early as 24 h after cRNA injection. Three days after injection, mNAT3 protein was mainly localized in the oocyte plasma membrane, whereas positive signals were undetectable with an affinity purified preimmune antibody (Fig. 2A). The anti-mNAT3 antibody did not detect positive signals in oocytes injected with water (data not shown).

The uptake of [^3H]-labeled L-alanine, L-glutamine, L-glutamate, L-histidine, and L-lysine, representing groups of zwitterionic, anionic, and cationic amino acids, was measured using two different concentrations of the substrates in *Xenopus* oocyte system. Compared to control water-injected oocytes, oocytes expressing mNAT3 exhibited uptake of L-alanine most strongly, followed by L-histidine and L-glutamine. A modest increase in L-lysine uptake was observed, while there was no significant increase in L-glutamate uptake (Fig. 2B). These results demonstrate that the most favorable substrate for mNAT3 is L-alanine followed by L-histidine and L-glutamine. mNAT3 has a similar transporting substrate selectivity as the other N-system transporters, mNAT, SN1 and mNAT2 [6,7,9] and the transporting pattern is almost identical to its orthologs hNAT3 and ATA3 [8,12].

To determine the substrate specificity of mNAT3, a competition assay in the presence of all 20 L-amino acids and the system A specific substrate, MeAIB¹ [3] was performed (Fig. 2C). As the most optimal substrate for mNAT3, L-alanine was selected as a model for the competition assay. In this assay, the rate of L-alanine uptake was determined in oocytes injected with mNAT3 cRNA in the presence of 5 mM nonradioactive-labeled 20 L-amino acids and MeAIB. The uptake of L-alanine at 50 μM was significantly inhibited by the following amino acids, listed in order of inhibitory potency: L-alanine > L-histidine > L-serine > L-glycine > L-asparagine > L-methionine. When 1 mM of L-alanine was used in the uptake assay in the presence of 5 mM nonradioactive-labeled 20 L-amino acids and MeAIB, the blocking of L-alanine uptake was less significant although the pattern of the inhibition sustained. System A specific substrate, MeAIB was unable to significantly block the transport of L-alanine, suggesting that mNAT3, is a system N amino acid transporter and is not a system A transporter.

mNAT3 is a Na^+ - and pH-dependent Low-affinity Transporter.

To characterize the transport properties of mNAT3, we examined the Na^+ -dependence and tolerance for Li^+ substitution of mNAT3 (Fig. 3A). In the presence of Na^+ buffer, L-alanine uptake into oocytes expressing mNAT3 was significantly greater than the uptake in the absence of Na^+ . Replacement of Na^+ by Li^+ partially recovered the stimulatory effect by Na^+ . Choline ion substitution for Na^+ led to a significant decrease in L-alanine uptake although there was 20% transport activity remaining. The 20% uptake rate observed in the absence of Na^+ could be due to the residual levels of Na^+ leaking from the injected oocytes or the transporting activity of

another system activated by the presence of mNAT3. To further confirm the Na⁺ dependence of the transport activities of mNAT3, the alanine uptake experiments with various concentrations of Na⁺ were performed (Fig. 3B). The increase in uptake rate is associated with an increase in Na⁺ concentration and reached maximum at the concentrations higher than 100 mM. These results demonstrate that L-alanine uptake mediated by mNAT3 is Na⁺-dependent and partially tolerates replacement of Na⁺ by Li⁺, which is consistent with the properties exhibited by system N transporters as previously described [6,30].

Extracellular pH in the range between 6.5 and 8.5 affected L-alanine uptake mediated by mNAT3. The results showed that uptake of L-alanine exhibited a pH-dependency increasing from low to high pH (Fig. 3C). To analyze the transport rate and substrate affinity of mNAT3, the concentration dependence of L-alanine uptake mediated by mNAT3 was investigated (Fig. 3D). At the lower concentration, the rate of uptake was an incremental function of concentration, whereas at higher concentrations, uptake was close to saturation. This observation indicates that L-alanine uptake in oocytes expressing mNAT3 behaved like a carrier-mediated transporter. The V_{\max} determined is 22 ± 7.1 pmol/min/oocyte and the K_m is 1.6 ± 0.3 mM. Compared to other mNATs analyzed in *Xenopus* oocytes [6,7], mNAT3 has a comparable substrate affinity (K_m), indicating a low-affinity transporter.

mNAT3 is Predominately Expressed in the Liver.

To elucidate the tissue-specific expression of mNAT3, Northern blots of equivalent amounts of RNA isolated from various tissues were probed with labeled mNAT3 and β -actin DNA fragments under high stringency condition (Fig. 4). Two bands in the approximate size of 4 kb and 2 kb were detected in mouse liver. The intactness of the β -actin band shown on the blots (Fig. 4, lower panel) and the proper intensity ratio of 28 S and 18 S ribosomal RNA (data not shown) excluded the possibility that the two bands detected by mNAT3 probe were caused by the degradation of RNA. Trace levels of mNAT3 expression were detected in muscle while there were no detectable signals in heart, kidney, brain, lung, small intestine, spleen, and thymus.

Expression of mNAT3 in Liver Hepatocytes Surrounding the Central Vein.

The molecular mass predicted by mNAT3 clone is around 60.6 KDa. Western blot analysis with an affinity-purified mNAT3 specific antibody revealed an immunoreactive protein

band at M_r of 80 kDa in liver samples (Fig. 5A, lane 2, arrow). The specificity of this protein band was further ascertained by the lack of detected immunoreactivity by probing with preimmune serum (Fig. 5A, lane 1) or by preincubating the mNAT3 antibody with the mNAT3 antigen (Fig. 5A, lane 3). Furthermore, an antibody for a nonrelated protein, connexin 43, did not compete for the binding of the mNAT3 antigen (data not shown). Expression of mNAT3 was detected in the liver with the aid of confocal microscopy. Immunofluorescence detection with antibody specific to mNAT3 demonstrated its localization to hepatocytes (Fig. 5B). The localization of mNAT3 appeared to be basolateral in the plasma membrane of hepatocytes surrounding the central vein (Fig. 5B, panel a). There is a graded decreased distribution of mNAT3 expression from the central vein to the portal tract (Fig. 5B, panel b). This specific distribution of mNAT3 in liver is consistent with a function of this protein in amino acid transport [31].

Insulin Induced an Increase in the Expression of mNAT3 and the Uptake of L-alanine in Hepatocyte-like H2.35 Cells.

The expression of mNAT3 in the forms of mRNA and protein was detected in hepatocyte-like H2.35 cells (Fig. 6A and B). The 4 kb form of mRNA is more dominant in H2.35 cells (Fig. 6A, lane 1) as compared to that from the liver (Fig. 6A, lane 2). An 80 kDa migrating protein band was detected with anti-mNAT3 antibody in hepatocyte-like H2.35 cells (Fig. 6B, lane 1), while no expression of mNAT3 protein was observed in neuroblastoma-like N2A cells (Fig. 6B, lane 2). Immunofluorescence of confocal microscopy of the cells shows that mNAT3 was localized at the plasma membrane (Fig. 6C, panel b, arrowheads).

Treatment with insulin increased the expression level of mNAT3, and this increase was directly associated with the concentration of insulin applied (Fig. 7A). This effect was observed even at 0.5 $\mu\text{g/ml}$ of insulin (Fig. 7A, lanes 1 and 2). Moreover, the insulin-induced stimulation of mNAT3 expression was dependent upon the length of the treatment period. As compared to the expression of the housekeeping protein β -actin, a significant increase of mNAT3 expression was observed after 4 h of insulin treatment becoming more profound at 8 h and sustaining up to 24 h of treatment (Fig. 7B). The increase in the amount of mNAT3 between the treatment of 8 to 24 h was less significant. This could be implied that longer period of the insulin treatment may not or stimulate less mNAT3 biosynthesis and existing intracellular protein pool of mNAT3 is

stabilized. Insulin also stimulated the uptake of L-alanine in H2.35 cells. Under the various insulin treatment periods, the uptake of L-alanine was gradually increased (Fig. 7C). The significant upregulation of the transport was observed 4 h after the treatment and the upregulation continued up to 24 h. An increase of L-alanine uptake was not caused by increased cell numbers since, as compared to control, the total number of cells were not altered under the identical treatments of insulin (data not shown). Together, these data suggest that the upregulation of mNAT3 expression by insulin is likely to lead to an increase of L-alanine uptake in hepatocytes.

Regulation of the Expression mNAT3 by Insulin-PI3 Kinase¹ Signaling

The insulin signaling cascade consists of two major downstream pathways: the MAP kinase pathway and the PI3 kinase pathway [32]. To determine which pathway is responsible for the stimulatory effect of insulin, we took advantage of two well-characterized specific inhibitors: PD98059 for MAP kinase pathway, and LY294002 for the PI3 kinase pathway [32]. The expression of Akt, a downstream effector of PI3 kinase, and MAP kinase in H2.35 cells was ascertained through the immunoblots with their corresponding antibodies (Fig. 8A). The activation of MAP kinase and Akt kinase exhibited through the phosphorylation on certain amino acid residues was determined using antibodies against phospho-MAP kinase (Thr202/Tyr204) and phospho-Akt kinase (Thr308) (Fig. 8A). As compared to the non-treated control (Fig. 8A, lane 1), the activation of both kinases was detected upon treatment with insulin (Fig. 8A, lanes 4). MAP kinase activation was inhibited with PD98059 (Fig. 8A, lane 3, blotted with antibodies for MAPK^(PThr202/Tyr204)). Treatment with the PI3 kinase inhibitors LY294002 (lane 2) attenuated Akt activation (Fig. 8A, lane 2, blotted with antibodies for Akt^(PThr308)). Similar inhibitory effect was also observed by another Akt inhibitor wortmannin (data not shown). Together, these results suggest that insulin activated MAP kinase and PI3 kinase signaling in H2.35 cells and the inhibitors PD98059 and LY294002 can efficiently block the activation of each signaling pathway.

The potent inhibitors PD98059 and LY294002 were adopted to dissect which downstream pathway(s) of insulin-activated signaling was involved in the up-regulation of the expression of mNAT3 in H2.35 cells (Fig. 8B). Consistent with the data shown in Fig. 7, insulin induced an increase in mNAT3 expression (Fig. 8B, comparing lanes 1 and 2). This increased

expression was blocked by the PI3 kinase inhibitor LY294002 (Fig. 8B, lane 3), but not by the MAP kinase inhibitor PD98059 (Fig. 8B, lane 4). The inhibition of insulin-induced upregulation of mNAT3 by the PI3 kinase inhibitor wortmannin was also observed (data not shown). These results suggest that the insulin-stimulated upregulation of mNAT3 in hepatocytes is likely to be mediated through the PI3 kinase signaling pathway.

DISCUSSION

In this study, we have identified and characterized the third member of the N-system amino acid transport family, mNAT3, expressed predominantly in the liver hepatocyte. Sequence and functional analyses show that mouse mNAT3 is related to the N-system transporters, mouse mNAT and mNAT2, and their orthologs [6-11,13,33]. The degree of amino acid homology between mNAT3, mNAT, and mNAT2 is 50-55%. The greater degree of divergence between mNAT3 and the other two mNATs is in the amino terminus of the transporter. Based on the predicted topology, mNAT3, like mNAT2, is a type 2 membrane protein with 10 predicted transmembrane segments, placing the amino and carboxyl termini in the cytoplasm. Among system N family members, mNAT3 and its orthologs are the largest in size with 547 aa and a large second intracellular loop domain.

The substrate selectivity of mNAT3 conforms that of the previously characterized N-system transport [3]. However, it is not sufficient to classify amino acid transporter systems solely on the basis of substrate selectivity since other systems, such as the system A, can also mediate Na⁺-dependent L-alanine uptake [34]. An important feature used to distinguish between these two systems is the amino acid and its related substrate transport competition assay. It has been generally accepted that inhibition by MeAIB and L-histidine can be used to define the system A and N, respectively [3]. The activity of L-glutamine transport mediated by mNAT3 can be effectively inhibited by the system N substrate L-histidine, but not by the system A substrate MeAIB. Therefore, we define mNAT3 as a system N instead of system A transporter. In the reported substrate transport assay of ATA3 in *Xenopus* oocytes, uptake of the A-system substrate MeAIB is less than 25 fold as compared to the uptake of L-alanine [12], which further supports our observation that the A-system substrate MeAIB is not preferred by mNAT3 and its

orthologs. Moreover, unlike L-alanine, MeAIB fails to induce currents in ATA3-expressing oocytes [12]. The weak uptake of MeAIB is also reported in human ATA3 expressing HRPE cells [13]. These data suggest that the functional features of mNAT3 closely match those of the N-system transporters.

In the substrate competition assay, although the inhibitory patterns of the substrate inhibition persist, the inhibitory effect by 1 mM L-alanine is less significant than the inhibition by 50 μ M. The possible explanation is that the K_m of other competing amino acids could be magnitude lower than L-alanine, therefore, five-folds more competing amino acids (5 mM) over alanine (1 mM) may not be sufficient to block alanine transport. The inhibition by 5 mM cold L-alanine is also less significant. This could be explained by the less difference in the uptake rate between 1 mM and 5 mM L-alanine as compared to the difference in uptake rate between 50 μ M and 5 mM substrate (referred to Fig. 3D). Our substrate transport assay suggests that L-histidine is transported about as well as glutamine, but in the substrate competition assay, L-histidine acts much more effectively as an inhibitor. This phenomenon could be interpreted by the facts that sites on the transporter responsible for substrate translocation, and for substrate binding and inhibition may be different. Although histidine displays similar substrate transport capability as glutamine, it may have a stronger binding affinity, as compared to glutamine, thus displaying stronger inhibition capability. The evidence of the discrepancy between substrate translocation and substrate inhibition has previously been reported for amino acid transporters LAT1 [35], prostaglandin transporter PGT [36], and organic transporter rOCT1 [37], and different amino acid residues responsible for substrate translocation and binding have been identified [36].

Northern blot analysis revealed the existence of two distinct mRNA species, 2 and 4 kb. Similar migrating patterns of mRNAs are also observed for rat ATA3, but only the single form of mRNA is reported for human hNAT3 and ATA3 [8,12,13]. These two forms of mRNAs may reflect multiple transcription start sites, alternatively spliced isoforms, or the existence of a relatively stable processing intermediate. As compared to liver samples, the 4 kb form of mRNA is more dominant in H2.35 cells, indicating that the ratio of the two mRNAs could be altered under different cell context as previously reported for the cationic amino acid transporter cat-1 gene [38]. The 60.6-kDa mass predicted by the DNA sequence (SWISS-PROT) is considerably smaller than the $M_r = 80$ kDa observed on a SDS-PAGE from liver and H2.35 cell samples. The differences between the expected and observed protein bands could be caused by

posttranslational modifications, such as glycosylation and phosphorylation. However, we failed to detect any conserved N-glycosylation consensus sites in the predicted extracellular loop regions. Moreover, deglycosylation or dephosphorylation treatment did not alter the mNAT3 migrating patterns (data not shown), suggesting that it is unlikely that both types of modifications can account for these differences. The discrepancy between predicted and measured molecular weight can be attributed to the structural complexity of membrane proteins and the anomalous migration of the protein in SDS-PAGE. The migration on SDS-PAGE is reported to differ from predicted levels in mNAT and in other proteins with multiple transmembrane segments such as the gap junction protein Cx45.6 [6,22].

Our results show for the first time that insulin at the molecular level regulates the expression and functions of a member of the mNAT family. We have identified the predominant expression of mNAT3 in liver and a weak expression in the muscle. mNAT3 is specifically localized in the basolateral membranes of hepatocytes and hepatocyte-like cells. Mouse H2.35 cells have been characterized to closely mimic primary hepatocytes with the expression of typical marker proteins and enzymes [39,40], therefore, serving as an ideal *in vitro* model for hepatocytes. We found that the treatment with insulin up-regulated L-alanine uptake and mNAT3 expression in H2.35 cells, but had no effect on cell proliferation. Moreover, this process was mediated by a PI3 kinase-dependent signaling pathway. A couple of previous studies, however, have shown that insulin induces the transport activities of systems A and N-like substrates [16,19] and the insulin-induced uptake was facilitated by the activation of PI3 kinase [19]. Likewise, a recent study reveals that the activation of N-system transport in burn injury is mediated by PI3 kinase signaling [41]. In contrast to these reports, stimulation of system A transport by amino acid starvation in cultured fibroblasts requires ERK1/2 instead of PI3 kinase activation [42]. Previous studies have implied that stimulation of system A-like substrate transport via the MAPK pathway is mediated through a slow mechanism dependent upon overall protein synthesis and cell proliferation [43,44]. However, we found that cell number was not up-regulated by long-term insulin treatment by insulin, whereas the expression of mNAT3 was increased. Our finding is consistent in part with another report that insulin stimulates system N activity in muscle cells by a mechanism that is characterized by its sensitivity to inhibition by cycloheximide, indicating the involvement of the biosynthesis of specific proteins [45]. Therefore, it appears that employment of different signaling transduction

pathways is dependent upon the types of stimulation and the ERK activation is not required for the insulin-induced transport stimulation.

Based on the specific expression pattern, transport function, and regulation by insulin, our results indicate that mNAT3 may be involved in the metabolism and physiological functions in the liver. A previous study [46] shows that, in the resting conditions, gluconeogenesis in isolated periportal hepatocytes is higher than perivenous hepatocytes when incubated with L-alanine. However, exercise results in a higher gluconeogenesis activity from L-alanine substrate in perivenous hepatocytes than in periportal hepatocytes. Therefore, the expression and function of mNAT3 in perivenous hepatocyte may partially be involved in the uptake of plasma alanine and glutamine to accommodate the activity of gluconeogenesis. Since insulin is known to inhibit gluconeogenesis in hepatocytes, the stimulation of the expression and function of mNAT3 by insulin is, thus, unlikely to be related to the activities of gluconeogenesis. Previous studies have shown that insulin can increase overall mRNA translation and protein biosynthesis [47]. It is likely that the insulin-stimulated uptake activity of mNAT3 may assist in providing more free amino acids inside the cells to meet the needs of a corresponding increase in protein synthesis. Further studies will shed light on the *in vivo* function and the regulation of mNAT3 in the liver.

Acknowledgments- We thank D. Adan-Rice and E. Sanchez for technical assistance. We also thank Dr. P. Camacho and Y. Li in the Department of Physiology at UTHSCSA for providing *Xenopus* oocytes for transport assay, Dr. V.C. Frohlich, director of UTHSCSA Confocal Imaging Core Facility for assistance with imaging, and members of Dr. J. Jiang's laboratory for the critical reading of the manuscript. The work is supported by Welch foundation grants (to J. X. J) and National Institute of Health (to J.X.J. and F.L).

REFERENCES

1. Christensen, H.N. (1990) Role of amino acid transporter and countertransport in nutrition and metabolism. *Physiol.Rev.* **70**, 43-77

2. Zorzano, A., Fandos, C. and Palacin, M. (2000) Role of plasma membrane transporters in muscle metabolism. *Biochem.J.* **349**, 667-688
3. Palacin, M., Estévez, R., Bertran, J. and Zorzano, A. (1998) Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol.Rev.* **78**, 969-1054
4. Castagna, M.A., Shayakul, C., Trotti, D., Sacchi, V.F., Harvey, W.R. and Hediger, M.A. (1997) Molecular characteristics of mammalian and insect amino acid transporters: implications for amino acid homeostasis. *J.Exp.Biol.* **200**, 269-286
5. Malandro, M.S. and Kilberg, M.S. (2000) Molecular biology of mammalian amino acid transporters. *Annu.Rev.Biochem.* **65**, 305-336
6. Gu, S., Roderick, H.L., Camacho, P. and Jiang, J.X. (2000) Identification and characterization of an amino acid transporter expressed differentially in liver. *Proc.Natl.Acad.Sci.USA* **97**, 3230-3235
7. Gu, S., Roderick, H.L., Camacho, P. and Jiang, J.X. (2001) Identification and characterization of an N-system amino acid transporter expressed in retina and its involvement in glutamine transport. *J.Biol.Chem.* **276**, 24137-24144
8. Gu, S., Adan-Rice, D., Leach, R.J. and Jiang, J.X. (2001) A novel human amino acid transporter, hNAT3: cDNA cloning, chromosomal mapping, genomic structure, expression and functional characterization. *Genomics* **74**, 262-272
9. Chaudhry, F.A., Reimer, R.J., Krizal, D., Barber, D., Storm-Mathisen, J., Copenhagen, D.R. and Edwards, R.H. (1999) Molecular analysis of system N suggests novel physiological roles in nitrogen metabolism and synaptic transmission. *Cell* **99**, 769-780
10. Fei, Y.-J., Sugawara, M., Nakanishi, T., Huang, W., Wang, H., Prasad, P.D., Leibach, F.H. and Ganapathy, V. (2000) Primary structure, genomic organization, and functional and electrogenic characteristics of human system N 1, a Na⁺- and H⁺-coupled glutamine transporter. *J.Biol.Chem.* **275**, 23707-23717

11. Varoqui, H., Zhu, H., Yao, D., Ming, H. and Erickson, J.D. (2000) Cloning and functional identification of a neuronal glutamine transporter. *J.Biol.Chem.* **275**, 4049-4054
12. Sugawara M., Nakanishi, T., Fei, Y.-J., Martindale, R.G., Ganapathy, M.E., Leibach, F.H. and Ganapathy, V. (2000) Structure and function of ATA3, a new subtype of amino acid transport system A, primarily expressed in the liver and skeletal muscle. *Biochim.Biophys.Acta* **1509** , 7-13
13. Hatanaka, T., Huang, W., Ling, R., Prasad, P.D., Sugawara, M., Leibach, F.H. and Ganapathy V. (2001) Evidence for the transport of neutral as well as cationic amino acids by ATA3, a novel and liver-specific subtype of amino acid transport system A. *Biochim.Biophys.Acta.* **1510**, 10-17
14. Haussinger, D. (1990) Nitrogen metabolism in liver: structural and functional organization and physiological relevance. *Biochem.J.* **267**, 281-290
15. Watford, M., Chellaraj, V., Ismat, A., Brown, P. and Raman, P. (2002) Hepatic glutamine metabolism. *Nutrition* **18** , 301-303
16. McDowell, H.E., Eysers, P.A. and Hundal, H.S. (1998) Regulation of System A amino acid transport in L6 rat skeletal muscle cells by insulin, chemical and hyperthermic stress. *FEBS Lett.* **44**, 15-19
17. Low, S.Y., Taylor, P.M., Hundal, H.S., Pogson, C.I. and Rennie, M.J. (1992) Transport of L-glutamine across sinusoidal membranes of rat liver. Effects of starvation, diabetes and corticosteroid treatment. *Biochem.J.* **284**, 333-340
18. Low, S.Y., Rennie, M.J. and Taylor, P.M. (1997) Signaling elements involved in amino acid transport responses to altered muscle volume. *FASEB J.* **11**, 1111-1117
19. Su, T.Z., Wang, M.H., Syu, L.J., Saltiel, A.R. and Oxender, D.L. (1998) Regulation of system A amino acid transport in 3T3-L1 adipocytes by insulin. *J.Biol.Chem.* **273**, 3173-3179

20. Tadros, L.B., Willhoft, N.M., Taylor, P.M. and Rennie, M.J. (1993) Effects of glutamine deprivation on glutamine transport and synthesis in primary culture of rat skeletal muscle. *Am.J.Physiol.* **265**, E935-E942
21. John, L.M., Lechleiter, J.D. and Camacho, P. (1998) Differential modulation of SERCA2 isoforms by calreticulin. *J.Cell Biol.* **142**, 963-973
22. Jiang, J.X., White, T.W., Goodenough, D.A. and Paul, D.L. (1994) Molecular cloning and functional characterization of chick lens fiber connexin45.6. *Mol.Biol.Cell* **5**, 363-373
23. Jiang, J.X., White, T.W., Paul, D.L. & Goodenough, D.A. (1995) in Intercellular communication through gap junctions. (Kanno, Y., Kataoka, K., Shiba, Y., Shibata, Y. & Shimazu, T., eds.), Molecular and functional characterization of lens fibers connexins. pp. 377-381, Elsevier, Amsterdam
24. Kim, J.W., Closs, E., Albritton, L.M. and Cunningham, J.M. (1991) Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. *Nature* **352**, 725-728
25. Taylor, P.M., Mackenzie, B.L.S.Y. and Rennie, M.J. (1992) Expression of rat liver glutamine transporters in *Xenopus laevis* oocytes. *J.Biol.Chem.* **267**, 3873-3877
26. Camacho, P. and Lechleiter, J. (1995) Calreticulin inhibits repetitive intracellular Ca^{2+} waves. *Cell* **82**, 765-771
27. Jiang, J.X. and Goodenough, D.A. (1998) Phosphorylation of lens fiber connexins by protein kinase C. *Eur.J.Biochem.* **255**, 37-44
28. Taylor, P.M., Hundal, H.S. and Rennie, M.J. (1989) Transport of glutamine in *Xenopus laevis* oocytes: relationship with transport of other amino acids. *J.Membr.Biol.* **112** , 149-157
29. Romero, M.F., Kanai, Y., Gunshin, H. and Hediger, M.A. (1998) Expression cloning using *Xenopus laevis* oocytes. *Methods Enzymol.* **296**, 17-52

30. Kilberg, M.S., Hahdlogten, M.E.C. and Istensen, H.N. (1980) Characteristics of an amino acid transport system in rat liver for glutamine, asparagine, histidine, and closely related analogs. *J.Biol.Chem.* **255**, 4011-4019
31. Burger, H.J., Gebhardt, R., Mayer, C. and Mecke, D. (1989) Different capacities for amino acid transport in periportal and perivenous hepatocytes isolated by digitonin/collagenase perfusion. *Hepatology* **9**, 22-28
32. Avruch, J. (1998) Insulin signal transduction through protein kinase cascades. *Mole.Cell.Biochem.* **182**, 31-48
33. Sugawara, M., Nakanishi, T., Fei, Y.-J., Huang, W., Ganapathy, M.E., Leibach, F.H. and Ganapathy, V. (2000) Cloning of an amino acid transporter with functional characteristics and tissue expression pattern identical to that of system A. *J.Biol.Chem.* **275**, 16473-16477
34. Barker, G. and Ellory, J.C. (1990) The identification of neutral amino acid transport systems. *Exp.Physiol.* **75**, 3-26
35. Uchino, H., Kanai, Y., Kim, D.K., Wempe, M.F., Chairoungdua, A., Morimoto, E., Anders, M.W. and Endou, H. (2002) Transport of amino acid-related compounds mediated by L-type amino acid transporter 1 (LAT1): Insights into the mechanisms of substrate recognition. *Mole.Pharmacol.* **61**, 729-737
36. Chan, B.S., Bao, Y. and Schuster, V.L. (2002) Role of conserved transmembrane cationic amino acids in the prostaglandin transporter PGT. *Biochemistry* **41**, 9215-9221
37. Mehrens, T., Lelleck, S., Cetinkaya, I., Knollmann, M., Hohage, H., Gorboulev, V., Boknik, P., Koepsell, H. and Schlatter, E. (2000) The affinity of the organic cation transporter rOCT1 is increased by protein kinase C-dependent phosphorylation. *J.Am.Soc.Nephrol.* **11**, 1216-1224
38. Aulak, K.S., Liu, J., Wu, J., Hyatt, S.L., Puppi, M., Henning, S.J. and Hatzoglou, M. (1996) Molecular sites of regulation of expression of the rat cationic amino acid transporter gene. *J.Biol.Chem.* **271**, 29799-29806

39. Enat, R., Jefferson, D.M., Ruiz-Opazo, N., Gatmaitan, Z., Leinwand, L.A. and Reid, L.M. (1984) Hepatocyte proliferation *in vitro*: Its dependence on the use of serum-free hormonally defined medium and substrate of extracellular matrix. *Proc.Natl.Acad.Sci.* **81**, 1411-1415
40. Dipersio, C.M., Jackson, D.A. and Zaret, K.S. (1991) The extracellular matrix coordinately modulates liver transcription factors and hepatocyte morphology. *Mole.Cell.Biol.* **11**, 4405-4414
41. Pawlik, T.M., Lohmann, R., Souba, W.W. and Bode, B.P. (2000) Hepatic glutamine transporter activation in burn injury: role of amino acids and phosphatidylinositol-3-kinase. *Am.J.Physiol.Gastrointest.Liver Physiol.* **278**, G532-G541
42. Franchi-Gazzola., Visigalli, R., Bussolati, O., Dall'Asta, V. and Gazzola, G.C. (1999) Adaptive increase of amino acid transporter system A requires ERK1/2 activation. *J.Biol.Chem.* **274**, 28922-28928
43. McGivan, J.D. and Pastor-Anglada, M. (1994) Regulatory and molecular aspects of mammalian amino acid transport. *Biochem.J.* **299**, 321-334
44. Longo, N., Franchi-Gazzola, R., Bussolati, O., Dall'Asta V., Foa, P.P., Guidotti, G.G. and Gazzola, G.C. (1985) Effect of insulin on the activity of amino acid transport systems in cultured human fibroblast. *Biochim.Biophys.Acta* **21**, 216-223
45. Tadros, L.B., Taylor, P.M. and Rennie, M.J. (1993) Characteristics of glutamine transport in primary tissue culture of rat skeletal muscle. *Am.J.Physiol.* **265**, E135-E144
46. Desy, F., Burelle, Y., Belanger, P., Gascon-Barre, M. and Lavoie, J.M. (2001) Effects of acute exercise on the gluconeogenic capacity of periportal and perivenous hepatocytes. *J.Appl.Physiol.* **91**, 1099-1104
47. Kleijn, M., Scheper, G.C., Voorma, H.O. and Thomas, A.A.M. (1998) Regulation of translation initiation by signal transduction. *Eur.J.Biochem.* **253**, 531-544

FIGURE LEGENDS:

Figure 1. Nucleotide and deduced amino acid sequences of mNAT3. A portion of a cDNA fragment was sequenced on both strands. The ORF of mNAT3 starting at nt 161 and ending at nt 1804 (underlined) encodes a protein with 547 amino acids. The derived amino acid sequence of mNAT3 with single letter code is shown on the lower line.

Figure 2. mNAT3 expression, amino acid transport, and competition analyses in *Xenopus* oocytes. *A.* Immunofluorescence of mNAT3. Frozen sections of *Xenopus Laevis* oocytes 3 days after injection with mNAT3 cRNA were immunolabeled with an affinity-purified anti-mNAT3 antibody or preimmune antibody and were examined by confocal microscopy at a scanning interval section of 0.5 μm . [Bar, 30 μm] *B.* Amino acid uptake rates in oocytes expressing mNAT3 against control, non-expressing oocytes. L-alanine, L-glutamine, L-glutamate, L-histidine, and L-lysine uptake rates were measured in uptake buffer containing 50 μM and 500 μM [^3H] L-labeled amino acid and incubated for 30 min ($n = 7$). *C.* Competitive inhibition by 20 L-amino acids and MeAIB on the uptake of L-alanine. The inhibition of 50 μM and 1 mM L-alanine uptake in oocytes expressing mNAT3 by 5 mM of 20 L-amino acids as well as MeAIB (Me). The percentage values after subtracting the controls injected with water are presented as 50 μM or 500 μM L-alanine uptake, defined as 100% ($n = 6$). A*, L-alanine uptake in the absence of non-labeled amino acids. All data are presented as mean \pm SEM.

Figure 3. Na^+ -, pH-, and concentration dependence analyses of mNAT3. *A.* Effect of Na^+ on the uptake of L-alanine mediated by mNAT3. Uptake of 50 μM [^3H] L-alanine was measured in the presence of Na^+ or in the presence of Li^+ buffer or choline $^+$ buffer to replace Na^+ buffer ($n = 5$). *B.* Na^+ dependence of L-alanine uptake. Uptake of L-alanine was measured in the presence of Na^+ at different concentrations of 0, 15, 30, 60, 90, 120, and 150 mM ($n = 5$). *C.* pH dependence of L-alanine uptake. Uptake of 50 μM [^3H] L-alanine was measured with Na^+ buffer under the indicated pH ($n = 5$). *D.* Concentration dependence of L-alanine uptake. Net uptake of L-alanine was measured at different concentrations (0 - 8 mM) of L-alanine in Na^+ buffer at pH 7.5. K_m of 1.6 ± 0.3 mM and V_{max} of 22 ± 7.1 pmol/min/oocyte for L-alanine were determined ($n = 6$). All data are presented as mean \pm SEM.

Figure 4. Northern blot analyses of tissue-specific expression patterns of mNAT3. Northern blots with equal amount of total RNAs from different mouse tissues were hybridized with [³²P] DNA probes of mNAT3 and β -actin under high-stringency condition. The size standards are indicated on the left.

Figure 5. Expression and localization of mNAT3 in the liver. *A.* Western blot detection of mNAT3 expression in the mouse liver. Crude membranes isolated from the liver were separated on 10% SDS-PAGE and immunoblotted with 1: 300 dilution of affinity-purified preimmune (lane 1), anti-mNAT3 (lane 2) antibodies, and anti-mNAT3 pretreated with mNAT3 antigen (lane 3). *B.* Immunofluorescence detection localized mNAT3 to the plasma membrane of hepatocytes surrounding the central vein (V) (a). The expression diminishes gradually toward the portal tract (T) (b). [(a) Bar, 20 μ m; (b) Bar, 200 μ m.]

Figure 6. Expression of mNAT3 in mouse hepatocyte-like cells. *A.* Northern blots with RNAs from H2.35 cells (lane 1) and mouse liver (lane 2) were hybridized with [³²P] DNA probe of mNAT3 and β -actin under high-stringency condition. *B.* Immunoblot of crude membranes isolated from H2.35 (lane 1) and N2A (lane 2) cells were labeled with 1:300 dilution of affinity purified anti-mNAT3 antibody. *C.* Immunofluorescence detection of mNAT3. Hepatocyte-like cells were immunolabeled with affinity purified anti-mNAT3 antibody (1:300 dilution) and the expression of mNAT3 was examined by fluorescence confocal microscopy (b). Phase-contrast image is shown in (a). Arrowheads point to the expression of mNAT3 at the cell borders.

Figure 7. Stimulatory effect of insulin on the expression of mNAT3 and the uptake of L-alanine in H2.35 cells. H2.35 cells were cultured in normal growth medium until they were confluent. Cells were incubated in serum free medium for 24 h [39] prior to the treatment of insulin. *A.* The expression of mNAT3 in cells treated with various concentrations of insulin. H2.35 cell cultures were treated in the presence (0.5 μ g/ml, lane 2; 1 μ g/ml, lane 3; 2 μ g/ml, lane 4; 4 μ g/ml, lane 5; 8 μ g/ml, lane 6; 16 μ g/ml, lane 7) and absence (lane 1) of insulin for 24 h. Immunoblots of crude membranes isolated from treated H2.35 cells were labeled with affinity purified anti-mNAT3 antibody (1:300). *B.* The expression of mNAT3 in cells treated with insulin for various periods of time. H2.35 cells cultures were treated in the presence (lanes 1, 3,

5, 7, and 9) and absence (lanes 2, 4, 6, 8, and 10) of 0.5 $\mu\text{g/ml}$ insulin for 0 h (lanes 1 and 2), 4 h (lanes 3 and 4), 8 h (lanes 5 and 6), 16 h (lanes 7 and 8), and 24 h (lanes 9 and 10). The immunoblots of isolated membrane from treated H2.35 cells were labeled with affinity purified anti-mNAT3 antibody (1:300) or monoclonal anti- β -actin (1:5000) antibody. *C.* The uptake of L-alanine by insulin treatment. H2.35 cells were pretreated with 0.5 $\mu\text{g/ml}$ insulin for 0 h, 4 h, 16 h, and 24 h, and the uptake analyses were performed for all treated cells under the identical length of the cultured period. The uptake of L-alanine was measured in uptake buffer containing 50 μM [^3H]-labeled L-alanine for 5 min ($n = 3$). ($*p < 0.05$; $**p < 0.005$).

Figure 8. LY294002 inhibited the stimulatory effect of insulin on mNAT3 expression. *A.* Analysis of the activation of kinases and the effects of specific inhibitors. H2.35 cells were preincubated in the absence (lanes 1 and 4) and presence of the MAP kinase inhibitor PD98059 (50 μM) (lane 3) or the PI3 kinase inhibitors LY294002 (50 μM) (lane 2) for 1 h, followed by treatment with (lanes 2-4) and without (lane 1) insulin (0.5 $\mu\text{g/ml}$) for another hour. The treated cells were lysed and immunoblotted with antibodies against phospho-p44/42 MAP kinase ($\text{MAPK}^{\text{(Thr202/Tyr204)}}$) and phospho-Akt kinase ($\text{Akt}^{\text{(PThr308)}}$). The blotted membranes were stripped and reblotted with antibodies against p44/42 MAP kinase (MAPK) and Akt. *B.* Expression of mNAT3 in cells treated with insulin and its signaling inhibitors. H2.35 cells were preincubated in the absence (lanes 1 and 2) and presence of LY294002 (50 μM) (lane 3) or PD98059 (50 μM) (lane 4) for 1 h and followed by treatment with (lanes 2-4) and without (lane 1) insulin for 24 h. Immunoblot was performed with a 1:300 dilution of affinity purified anti-mNAT3 antibody and a 1:5000 dilution of monoclonal anti- β -actin antibody.

Footnotes:

¹Abbreviations used: DEPC, diethyl pyrocarbonate; FITC, fluorescein isothiocyanate; GST, glutathione S-transferase; IPTG, isopropyl β -D-D-thiogalactoside; MAP kinase, mitogen-activated protein kinase; MeAIB, N-methylamino-isobutyric acid; mNAT, mouse N-system

amino acid transporter; PI3 kinase, phosphatidyl inositol-3-kinase; ORF, open reading frame; RT-PCR, reverse transcription-polymerase chain reaction; kb, kilobase(s).

²The nucleotide sequence reported in this paper has been submitted to the GenBankTM/ EBI Data Bank with accession number AB055004.

Fig. 1

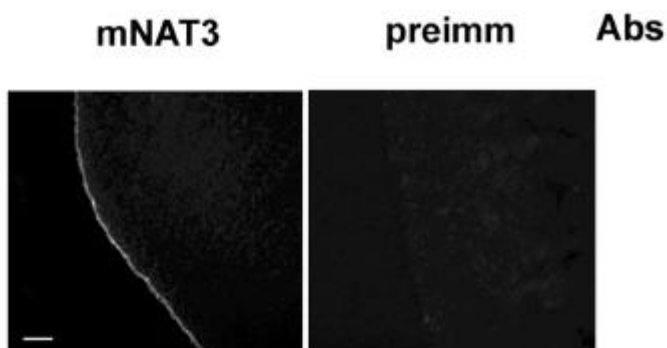
```

1 gctgaattctaggtctgcttccacatcttgaatgttttta
tccccatgaacagagtgttctgagcgtcaacacaaagtggaagagcctcgagctgaaggcgctgtgtattatgtgactgaagactctt
tctgtggacagggaaagggctgtgaggtcaaattggaccccatggaactgaaaagactcttttctgtggacagggaaagggctgtgaggtcaa
161 atggaccccatggaactgaacaacgtcagcatcgaacccgacggagacagctgcagcggggacagtattcaggacagctacaccggcatg
M D P M E L N N V S I E P D G D S C S G D S I Q D S Y T G M
251 gaaaactccgacaaggacgccatgaacagccaatttgctaataagatgccgaaagtcagaagttcctgacaaatgggttttagggaag
E N S D K D A M N S Q F A N E D A E S Q K F L T N G F L G K
341 aagaagctagccgattacgcggatgagcatcacccctggaatgacttcccttgggaatgtcctcatttaacctgagcaacgccatcatgggc
K K L A D Y A D E H H P G M T S F G M S S F N L S N A I M G
431 agtgggatcttaggcttgcctatgccatggccaacaccgggatcatcctttttataatcatgctgcttactgtggcaataactctcgctc
S G I L G L S Y A M A N T G I I L F I I M L L T V A I L S L
521 tactcggttcaccttttctgctgaagacagccaaggaaggagggtctctaataatgaaaaattgggcgagaaagcatttggatggcctggg
Y S V H L L L K T A K E G G S L I Y E K L G E K A F G W P G
611 aaaattggagccttcatctctattacaatgcagaacattggagccatgtcaagctacctcttcatcattaagtacgaactgcctgaagta
K I G A F I S I T M Q N I G A M S S Y L F I I K Y E L P E V
701 atcagagcattcatgggacttgaagaaaacactggggaatggtacctcaacggcaactacctcgtcttatttgtgtccgtggggatcatc
I R A F M G L E E N T G E W Y L N G N Y L V L F V S V G I I
791 ctcccgctctctctccttaaaaatttaggctaccttggctacaccagtggttttctctctcctgcatgggtgttttctgctcagtggtg
L P L S L L K N L G Y L G Y T S G F S L S C M V F F V S V V
881 atttacaataaaattccaaattccctgcccctctgctgctctggatcacaacaacggaaatctgacgttcaacaacacacttccgattcac
I Y K K F Q I P C P L P A L D H N N G N L T F N N T L P I H
971 atgatctcgctgcctaatactgactcggagagctcgggtgtgaacttcatgatggattacgctcaccacaaccagctgggctggatgagaag
M I S L P N D S E S S G V N F M M D Y A H H N P A G L D E K
1061 caggctcgcaggccctcttcacagcaacggcgtggagtacgaagcccagggtgctgagaaaatgccaacaaaatactttgtgttcaattcc
Q V A G P L H S N G V E Y E A Q G A E K C Q P K Y F V F N S
1151 cggacggcctatgcaatcccaatccctggcttttctgttctgctgccaccctgaggtccttcccatctacagcgagcttaagatcgatcc
R T A Y A I P I L A F A F V C H P E V L P I Y S E L K D R S
1241 cgcagaaagatgcagacgggtgtccaacatttccatctcaggcatgtcgtcatgtaccttcttgcggccctctttgggttatctgagcttc
R R K M Q T V S N I S I S G M L V M Y L L A A L F G Y L S F
1331 tacggggacggttgaagacgagctgctgcatgcttacagcaaggtctacacatttgatagcgctcttctcatgggtgctgctggcagtcctg
Y G D V E D E L L H A Y S K V Y T F D T A L L M V R L A V L
1421 gtggcagtgacactgaccgtgcccatcgtgctgttcccgatccgtacttccggtgatcacactgctgtttccaaggaaacccttcagctgg
V A V T L T V P I V L F P I R T S V I T L L F P R K P F S W
1511 ctgaagcatttccggatcgtgcaatcatcatcgcactcaacaacatcctggatcctcctgctgctaccatcaatacatctttggattc
L K H F G I A A I I I A L N N I L V I L V P T I K Y I F G F
1601 ataggggcttcttctgccactatgctgattttcattcttccggctgcgttttatctcaagctcgtcaagaaagaaccttaagatcaccc
I G A S S A T M L I F I L P A A F Y L K L V K K E P L R S P
1691 cagaagattggggcttttggcttctccttgtgactggaattattttcatgatgggaagcatggcgtcattatactcgactggatctacaac
Q K I G A L V F L V T G I I F M M G S M A L I I L D W I Y N
1781 ccgccgaatcccaatcaccactaaatcccggggagacgcgtctccactggaaacagctgaaattgtctgaaggacatttttagttgtcttga
P P N P N H H *
1871 ttgggatgttagtctgaggaattagcaagattccaaagacgtttttctagctctatcatgggatacttgtggaagagaaaattatggggt
1961 ttgttgggaatgggttttgggttgggaatgggtgaaggatgcattaaaaattctgtggcacacatttta

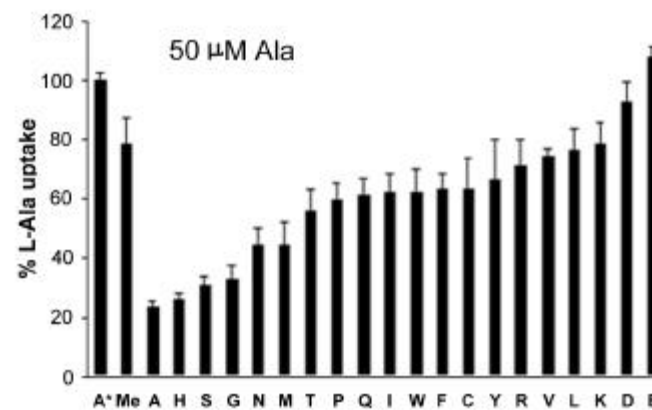
```

Fig. 2

A



C



B

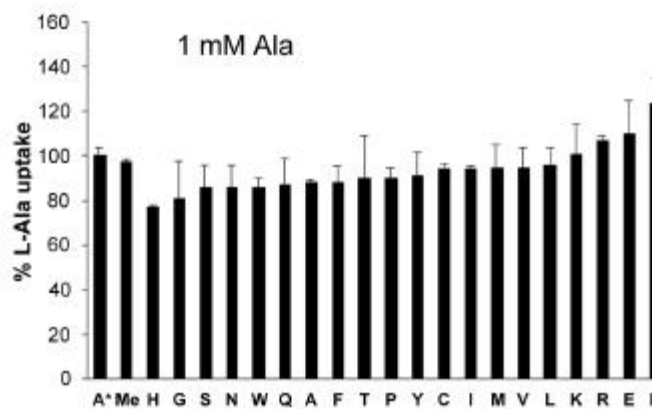
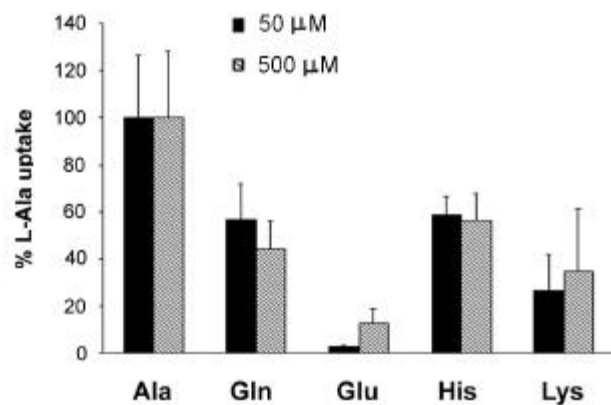
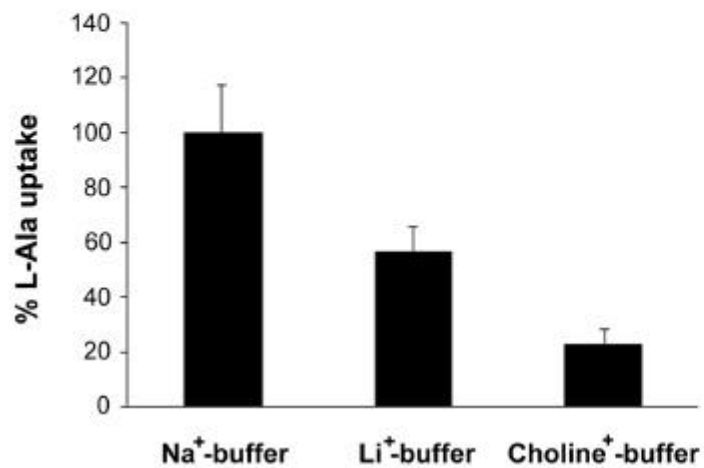
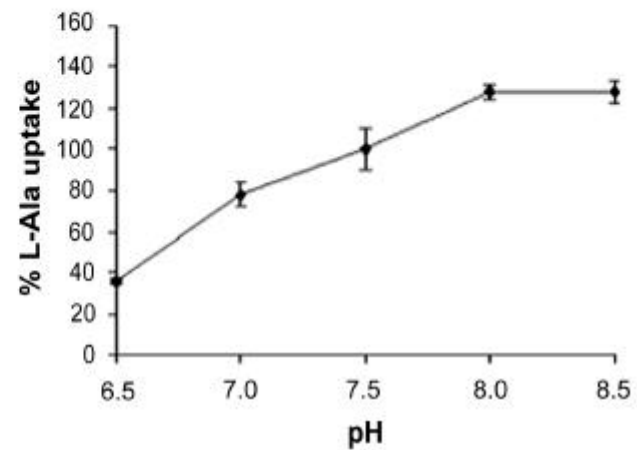


Fig. 3

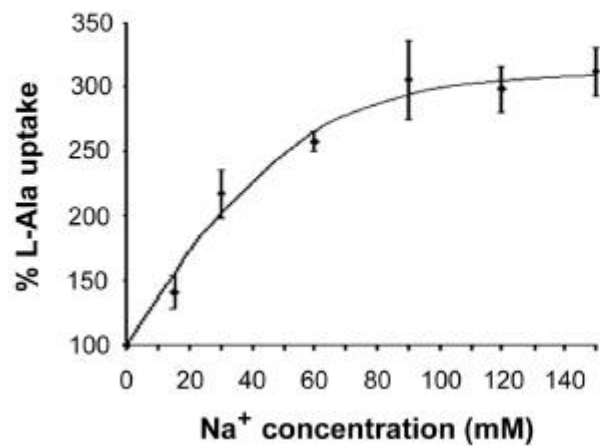
A



C



B



D

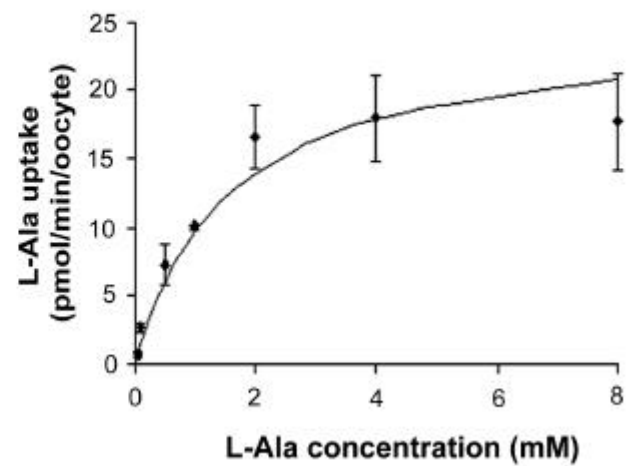


Fig.4

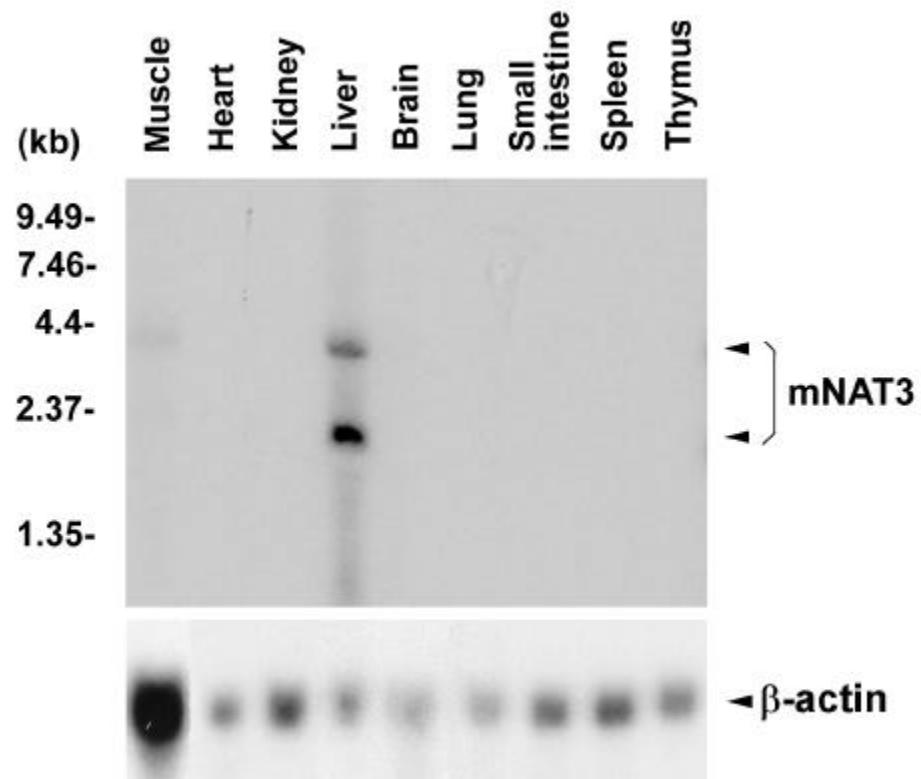


Fig. 5

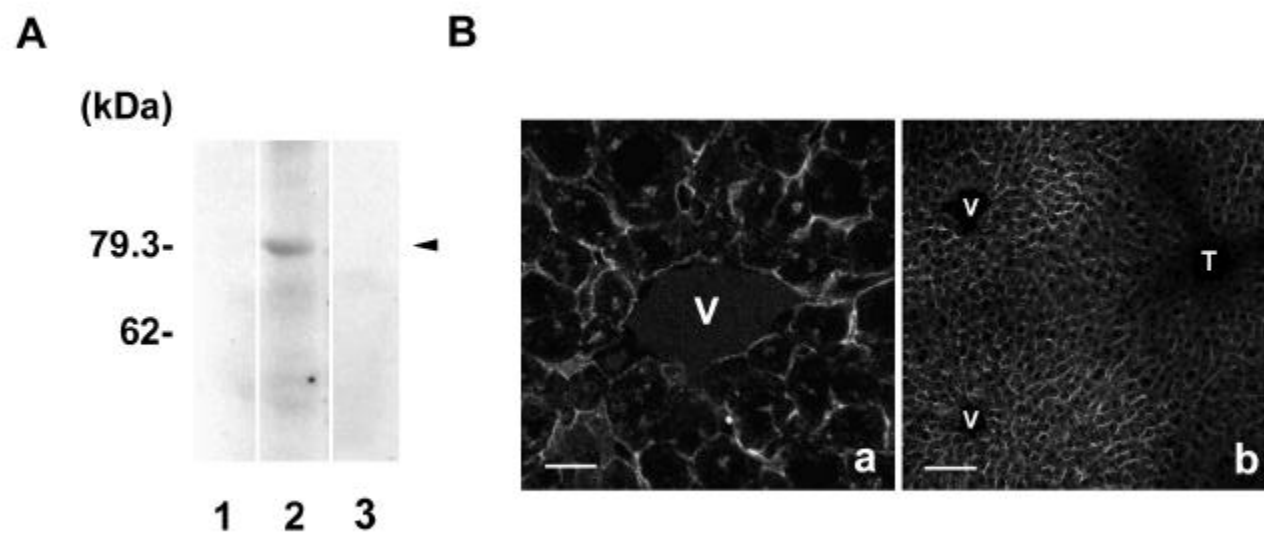


Fig. 6

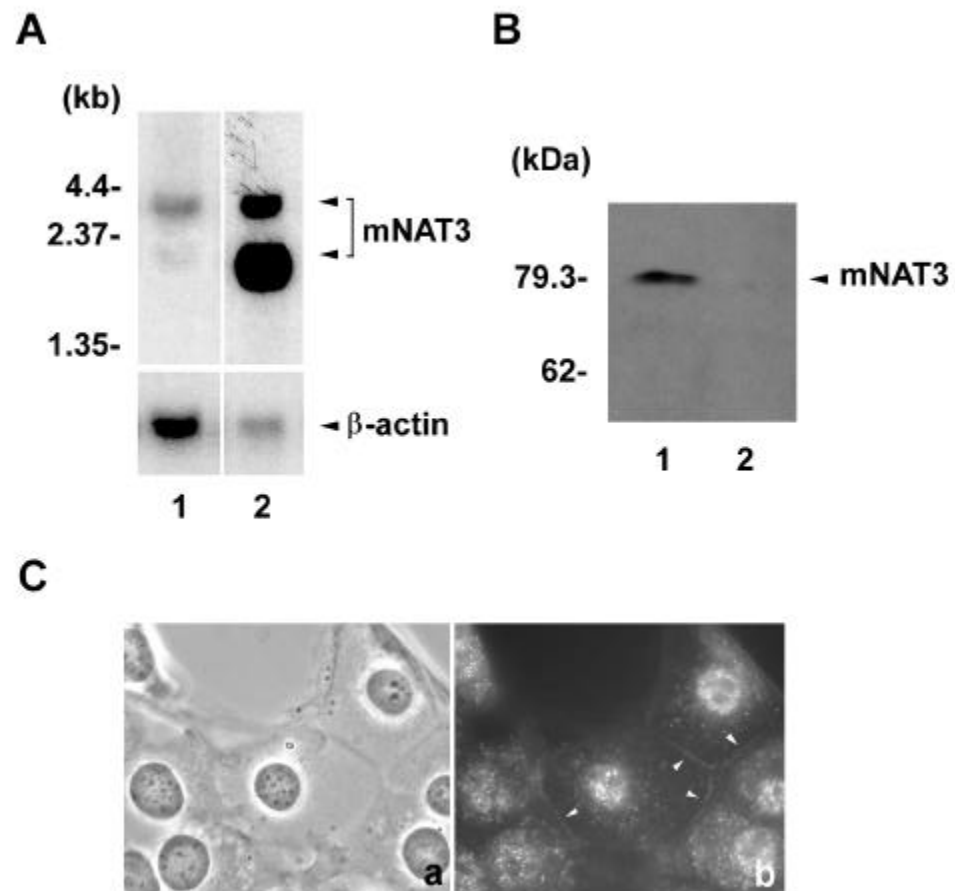
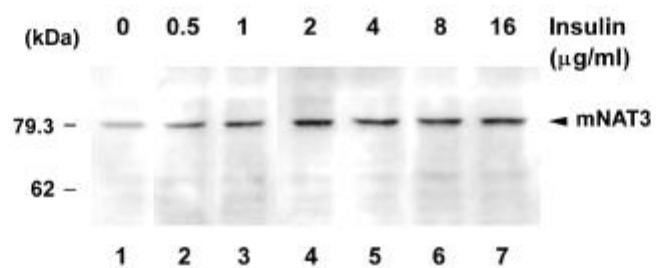
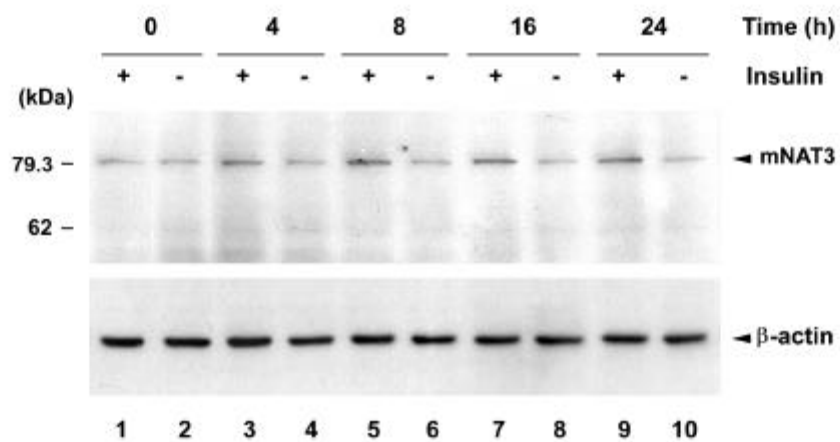


Fig. 7

A



B



C

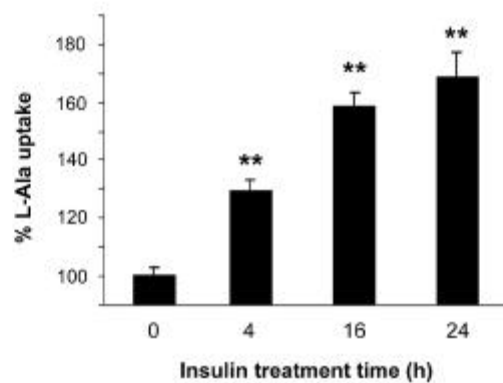


Fig. 8

