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High-performance affinity chromatography method for identification of L-arginine interacting factors using magnetic nanobeads

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ABSTRACT: L-Arginine exhibits a wide range of biological activities through a complex and highly regulated set of pathways that remain incompletely understood at both the whole-body and the cellular levels. The aim of this study is to develop and validate effective purification system for L-arginine interacting factors (AIFs). We have recently developed novel magnetic nanobeads (FG beads) composed of magnetite particles/glycidyl methacrylate (GMA)–styrene copolymer/covered GMA. These nanobeads have shown higher performance compared with commercially available magnetic beads in terms of purification efficiency. In this study, we have newly developed L-arginine methyl ester (L-AME)-immobilized beads by conjugating L-AME to the surface of these nanobeads. Firstly, we showed that inducible nitric oxide synthase, which binds and uses L-arginine as a substrate, specifically bound to L-AME-immobilized beads. Secondly, we newly identified phosphofructokinase, RuvB-like 1 and RuvB-like 2 as AIFs from crude extracts of HeLa cells using this affinity chromatographic system. The data presented here demonstrate that L-AME-immobilized beads are effective tool for purification of AIFs directly from crude cell extracts. We expect that the present method can be used to purify AIFs from various types of cells. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: affinity chromatography; magnetic nanobeads; L-arginine interacting factors; L-arginine methyl ester

Introduction

L-Arginine is classified as a semi-essential amino acid because the ability of the body to synthesize sufficient quantities to meet its needs varies according to developmental age and the incidence of disease or injury (Morris, 2006). The sources of free L-arginine within the body are dietary protein, turnover of body proteins, and endogenous synthesis. L-Arginine is synthesized from citrulline by the sequential action of the cytosolic enzymes arginosuccinate synthetase and arginosuccinate lyase. On the other hand, L-arginine can be catabolized by four sets of enzymes in mammalian cells: nitric oxide synthases (NOS), arginase, arginine–glycine amidinotransferase and arginine decarboxylase, and be converted to a variety of biologically active compounds such as nitric oxide (NO), creatine phosphate, agmatine, polyamines, ornithine and citrulline (Morris, 2006). L-Arginine has many effects in the body that include modulation of immune function (Angele *et al.*, 2002), wound healing (Stechmiller *et al.*, 2005), hormone secretion (Fisker *et al.*, 1999), vascular tone (Koizumi *et al.*, 1994), endothelial function (Schlaich *et al.*, 2004) and insulin secretion (Weinhaus *et al.*, 1997). Thus, L-arginine is a biologically active dietary compound that mediates numerous physiological activities directly or via metabolized products (Tong and Barbul, 2004).

Metabolic enzymes that use L-arginine as substrates are well-known L-arginine interacting factor (AIFs); however, much less is known about the AIFs that directly mediate the physiological effects of L-arginine. Identification of AIFs is necessary in order to study arginine signaling, because most signaling molecules bind to the proteins that exist on the cell surface or in the cells to mediate the biochemical reactions. There is, however, no efficient tool to purify and identify AIFs. We had previously developed nanobeads composed of a GMA-covered GMA-styrene copolymer core (SG beads, 200 nm in diameter) (Kawaguchi *et al.*, 1989). These nanobeads have several advantages over conventional

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Abbreviations used: AIF, L-arginine interacting factor; EGDE, ethyleneglycol diglycidyl ether; GMA, glycidyl methacrylate; iNOS, inducible nitric oxide synthase; L-AME, L-arginine methyl ester; L-NAME, *N*ω-nitro-L-arginine methyl ester; NHS, *N*-hydroxysuccinimide.

affinity purification supports: (1) their lack of pores results in efficient removal of residual proteins during the washing steps, as well as easy access for target proteins to the fixed ligands; (2) the small diameter of the beads (200 nm) provides a large surface area (1 g of the beads have a surface area of 20 m^2), giving the beads a high capacity to immobilize ligands; (3) the chemical and physical stability of the beads permits coupling of ligands in the presence of a wide range of organic solvents. This enables the rapid and efficient purification of ligand-binding proteins, including transcription factors (Inomata *et al.*, 1992; Wada *et al.*, 1995), drug receptors (Shimizu *et al.*, 2000; Hiramoto *et al.*, 2002). Based on SG beads, we have recently developed novel magnetic nanobeads (FG beads, 200 nm in diameter) composed of magnetite particles/GMA–styrene copolymer/covered GMA (Nishio *et al.*, 2008). FG-beads show higher performance compared with commercially available magnetic beads, adembeads (200 nm, ADEMTECH), nanomag-D (130 nm, micromod) and Dynabeads (2.8 μm, Invitrogen), in terms of purification efficiency of target proteins (Nishio *et al.*, 2008).

To establish an effective purification system for AIFs, we have newly developed L-arginine analog-immobilized nanobeads. In this report, firstly we showed that L-AME is a suitable compound for purification of AIFs. Secondly, we confirmed that L-AME-immobilized beads specifically bind AIFs. Lastly, we successfully identified new AIFs using L-AME-immobilized beads. Here we show that this system is very effective for purification of AIFs directly from crude cell extracts.

Experimental

Materials

L-Arginine, L-AME, *Nω*-nitro-L-arginine methyl ester (L-NAME) and chymotrypsin were purchased from Sigma-Aldrich (St Louis, MO, USA). 2,3-Diaminonaphthalene, succinic anhydride, triethylamine, dithiothreitol and iodoacetamide were purchased from Nacalai Tesque (Kyoto, Japan). L-Phenylalanine and ethyleneglycol diglycidyl ether (EGDE) were purchased from Wako Chemicals (Osaka, Japan). *N*-Hydroxysuccinimide (NHS) was obtained from Peptide Institute Inc. (Osaka, Japan). Trypsin was obtained from Promega (Madison, WI, USA). HeLa cells and NIT-1 cells were obtained from American Type Culture Collection (Manassas, VA, USA). 293FT cells were obtained from Invitrogen (Carlsbad, CA, USA).

Insulin Production Analysis

L-Arginine- or L-AME-stimulated insulin secretion was determined as previously described (Weinhaus *et al.*, 1997). Briefly, after cultivation of NIT-1 cells, medium was replaced with perfusion solution (10 mM HEPES-NaOH pH 7.4, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose) for 10 min. The final concentration (0, 0.2, 0.6, 2.0, 20.0 mM) of L-Arginine or L-AME was added to the cells for 10 min and perfusion solution was collected. Insulin concentration in perfusion solution was measured by insulin ELISA kit according to the protocol of manufacture (Shibayagi, Gunma, Japan). The values are reported as means ± SEM for four independent experiments.

NO Production Analysis

Expression vectors of iNOS was transfected to 293FT cells (5.0×10^5) with over 80% transfection efficiency. At 24 h after transfection, conditioned medium was collected and NOS activity was determined as previously described (Misko *et al.*, 1993). Briefly, 10 μL of freshly prepared 2,3-diaminonaphthalene (50 μg/mL in 620 mM HCl) was added to 100 μL of conditioned medium and mixed immediately. After a 10 min incubation at room temperature, the reaction was terminated with 5 μL of 2.8 M NaOH. Formation of the 2,3-diaminonaphthotriazole was measured using a fluorescent plate reader using an excitation wavelength of 365 nm and an emission wavelength of 450 nm. The values are reported as means ± SEM. Statistical significance ($p < 0.005$) was determined by unpaired Student's *t*-test (STATVIEW).

Preparation of Radiolabeled Recombinant Proteins

T7 promoter tagged DNA fragments of various cDNA (iNOS, PPARγ, PFK, RBL1, RBL2) were amplified by PCR with two primers; T7 promoter fused to 5′ sequence of each cDNA and polyT fused to 3′ complimentary sequence of each cDNA. These DNA fragments were used to synthesize ^{35}S-radiolabeled recombinant proteins in a coupled transcription/translation system according to the protocol of manufacture (Promega, Madison, WI, USA).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the protein samples from chymotrypsin digestion analysis and L-arginine binding analysis, and to evaluate the affinity purified AIFs as previously described (Laemmli, 1970). Samples were diluted in SDS-PAGE loading buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 100 mM β-mercaptoethanol, 10% glycerol, 0.01% bromophenolblue) and denatured at 98°C for 5 min. Samples were applied in a 5–20% gradient precast gel (Wako, Osaka, Japan), and run at 200 V for 60 min using DPE-1020 Cassette Electrophoresis Unit (Daiichi, Tokyo, Japan).

Chymotrypsin Digestion Analysis

The protease digestion assay was performed as previously described with minor modifications (Allan *et al.*, 1992). Briefly, radiolabeled proteins (iNOS, PFK, RBL1, RBL2) were mixed with 100 μM compound (L-arginine, L-AME or L-phenylalanine) for 10 min on ice, then partially digested with different concentration of chymotrypsin (0, 1, 3, 10 μg/mL) for 10 min on ice. The protease digestions were terminated by the addition of SDS-PAGE loading buffer and boiling for 5 min. The products of the digestion were separated by SDS-PAGE. The gels were dried, and autoradiography was then performed to visualize the radiolabeled digestion products.

Preparation of L-AME-immobilized Beads

FG beads were prepared as previously described (Nishio *et al.*, 2008). Epoxy groups on FG beads were aminolysed by NH_4OH and coupled to EGDE to produce FGNEGDE beads (Shimizu *et al.*, 2000). Epoxy groups on FGNEGDE beads were aminolysed by NH_4OH (FGNEGDEN), carboxylated with succinic anhydride (FGNEGDENS) and activated with NHS to produce NHS-activated FGNEGDENS beads (Ohtsu *et al.*, 2005).

NHS-activated FGNEGDENS beads (2.5 mg) were incubated with 1.5 mM L-AME in 500 μL of DMSO containing 10% triethylamine at 37°C for 3 h. Released NHS during the coupling reaction was collected together with unbound L-AME. Coupling yield was determined by separating NHS released from the beads on a Symmetry C_{18} cartridge, 5 μm (Waters, Milford, MA, USA). Usually 40% of L-AME was immobilized onto the beads. The amount of L-AME immobilized onto the beads can be controlled by the amount of L-AME added into the immobilization reaction. Unreacted NHS-activated carboxylate was masked with 1 M 2-ethanolamine (pH 8.0) at 4°C for 24 h. L-AME-immobilized beads were suspended in distilled water and stored at 4°C until use.

L-Arginine Binding Analysis

L-AME-immobilized beads or control beads (200 μg) were equilibrated with binding buffer (20 mM HEPES–NaOH, pH 7.9, 10% glycerol, 200 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM EDTA, 1 mM DTT, and 0.2 mM PMSF) and incubated with 200 μL of the radiolabeled proteins (iNOS, PPARγ, PFK, RBL1, RBL2) at 4°C for 4 h using RT-50 rotator (15 rpm, TAITEC,

Saitama, Japan). After washing with binding buffer, bound proteins were eluted by boiling for 5 min with SDS-PAGE sample buffer. Eluates and inputs were subjected to SDS-PAGE. The gels were dried, and autoradiography was then performed to visualize the radiolabeled proteins.

Affinity Purification of AIFs

Cytoplasmic extracts of HeLa cells were prepared from 4 L culture as previously described (Dignam *et al.*, 1983). L-AME-immobilized beads or control beads (200 µg) were equilibrated with binding buffer (20 mM HEPES–NaOH, pH 7.9, 10% glycerol, 200 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM EDTA, 1 mM DTT, and 0.2 mM PMSF) and incubated with 200 µL of the cytoplasmic extracts at 4°C for 4 h using RT-50 rotator (15 rpm, TAITEC, Saitama, Japan). After washing with binding buffer, bound proteins were eluted with binding buffer containing 100 mM L-Arginine.

Mass Spectrometry Analysis of AIFs

Affinity purified AIFs were separated by SDS-PAGE and gels subjected to silver staining as previously described (Shevchenko *et al.*, 1996). The specific protein bands were excised, reduced with 10 mM dithiothreitol followed by alkylation with 55 mM iodoacetamide. Band slices were digested with trypsin (12 µg/mL) overnight and desalted with ZipTip C_{18} (Millipore, Billerica, MA, USA). The extracted peptides were then separated via nanoflow liquid chromatography (LC) (Paradigm MS4, AMR, Tokyo, Japan) using a reverse-phase C_{18} column (Magic C18, AMR, Tokyo, Japan). The LC eluent was coupled to a micro-ionspray source attached to a LCQ Advantage MAX mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA). All MS/MS spectra were searched using the TurboSEQUEST algorithm within the BioWorks 3.2 software (Thermo Electron Corporation, Waltham, MA, USA).

Results and Discussion

Evaluation of L-AME Activity (Figs 1 and 2)

To identify AIFs, several L-arginine related compounds were screened and L-AME was selected as the candidate compound (data not shown). L-Arginine is one of the strong insulin secretogogues (Kahn *et al.*, 2005). However, the mechanism of its stimulation is not well understood, and the AIFs are not identified. Actually L-arginine stimulated insulin production in pancreatic beta NIT-1 cells (open circle in Fig. 1A). The maximal stimulation of insulin production was achieved at a 2 mM of L-arginine. The amount of insulin production stimulated at 20 mM L-arginine was less than that stimulated at 2 mM L-arginine; however, the decrease was not statistically significant. L-AME also stimulated insulin production in a dose-dependent manner (solid square with dotted line, Fig. 1A). L-AME as well as L-arginine is a strong inducer for the insulin production.

NO and citrulline is converted from L-arginine by NOS. It has been reported that L-NAME binds to NOS with high affinity, and behaves as the binding antagonist of NOS (Dawson *et al.*, 1991). Actually, L-NAME suppressed the production of the NO metabolites nitrite and nitrate (triangles in Fig. 1B). L-AME also suppressed the production of the NO metabolites (squares in Fig. 1B). L-AME as well as L-NAME is the binding antagonist of NOS.

In the case of nuclear receptors that are ligand-dependent transcription factors, the conformational change due to ligand-binding has been shown to affect the protease sensitivity (Allan *et al.*, 1992; Berger *et al.*, 1996; Elbrecht *et al.*, 1999). *In vitro* translated iNOS was partially digested by chymotrypsin without and with L-arginine, L-AME, or L-phenylalanine (Fig. 2). Addition of L-arginine reduced the chymotrypsin sensitivity of iNOS compared with the negative control (no addition or L-phenylalanine addition). L-AME addition to the reaction also reduced the chymotrypsin sensitivity of iNOS similarly to L-arginine. Each of these two reagents, L-arginine and L-AME, does not directly reduce the chymotrypsin activity, because the cymotrypsin sensitivity of

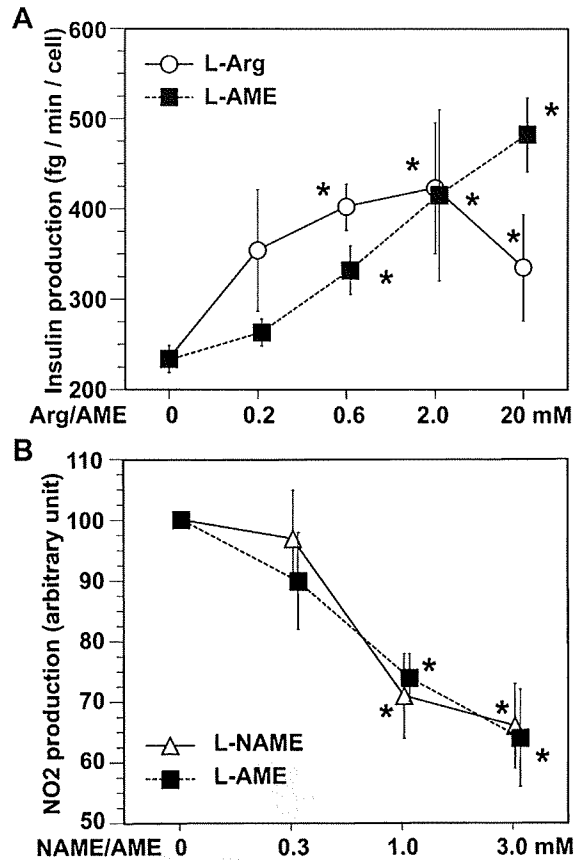

Figure 1. Evaluation of L-AME activity. (A) L-AME shows the similar activity to L-arginine for stimulation of insulin secretion. Insulin production from NIT-1 cells was measured in response to indicated (0, 0.2, 0.6, 2.0 and 20 mM) concentration of L-arginine (open circles) and L-AME (solid squares with dot line). (B) L-AME shows the similar activity to L-NAME for reducing of NO metabolites production. NO metabolite production was measured with indicated (0, 0.3, 1.0 and 3.0 mM) concentration of L-NAME (open triangles) and L-AME (solid squares with dotted line). *Statistically significant different from control (0 mM), $p < 0.01$.

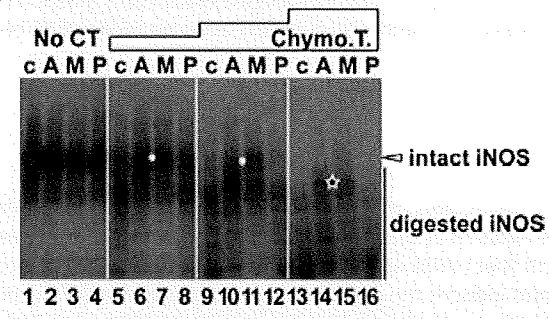

Figure 2. L-AME and L-arginine change iNOS sensitivity to chymotrypsin digestion. *In vitro* radiorabeled iNOS was partially digested by chymotrypsin without (lanes 1, 5, 9 and 13), with L-arginine (lanes 2, 6, 10 and 14), L-AME (lanes 3, 7, 11 and 15) or L-phenylalanine (lanes 4, 8, 12 and 16). Digested samples were analyzed in a 5–20% gradient SDS-PAGE. The open circles indicate the protease-resistant fragments of intact iNOS. The solid star indicates the protease-resistant fragment of digested iNOS.

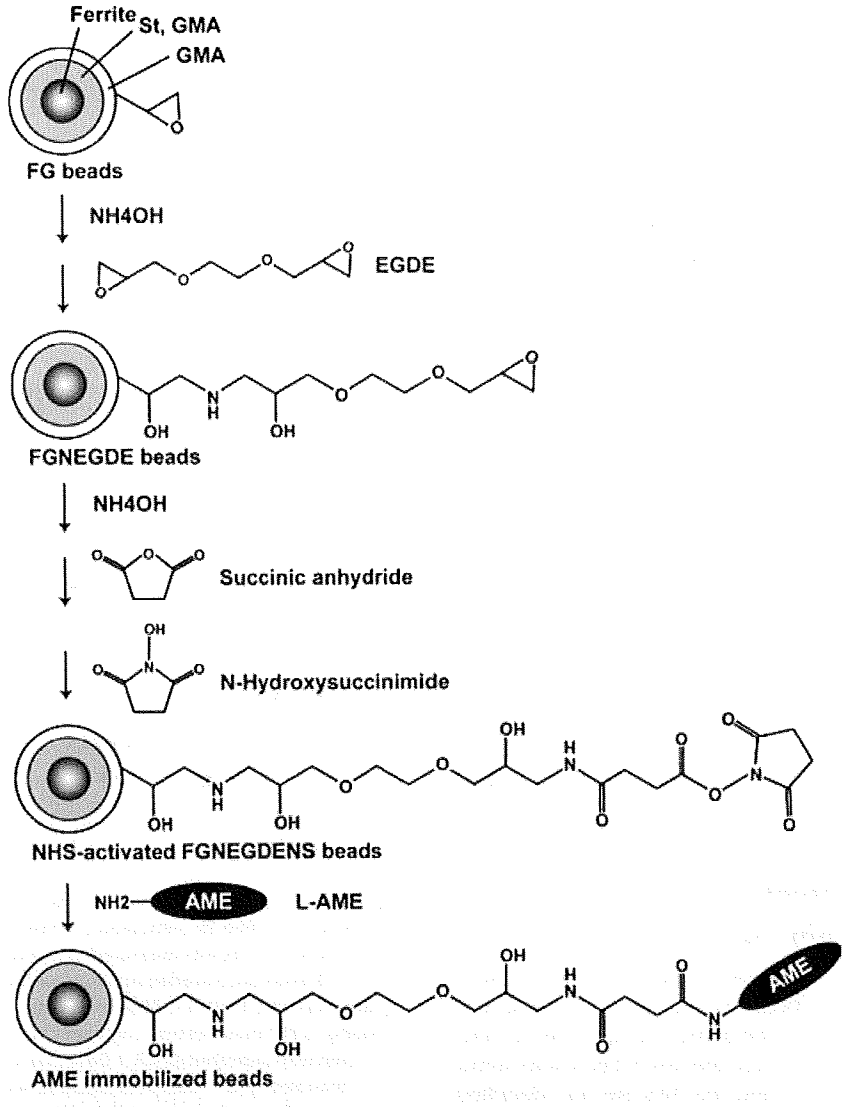

Figure 3. Preparation of L-AME-immobilized beads. Preparation of L-AME-immobilized beads. Epoxy groups on FG beads were aminolysed by NH_4OH and coupled to EGDE to produce FGNEGDE beads. Epoxy groups on FGNEGDE beads were aminolysed by NH_4OH, succinylated with succinic anhydride and activated with NHS to produce NHS-activated FGNEGDENS beads. NHS-activated FGNEGDENS beads were then coupled with amino groups of L-AME.

PPARγ, which does not bind to these reagents, has not changed by the addition of these reagents (data not shown). These results suggest that L-AME as well as L-arginine binds to iNOS protein *in vitro* and induces the conformational change of iNOS; in consequence, the accessibility of chymotrypsin to iNOS is changed and the reduction of the chymotrypsin sensitivity of iNOS is detected. Taken together L-AME was selected as the suitable compound immobilized to nanobeads for efficient purification of AIFs.

Preparation of L-AME-immobilized Beads (Fig. 3)

To purify AIFs, we prepared L-AME-immobilized beads. We selected L-AME, not L-arginine, as the ligand compound in order to avoid the self-condensation reaction of L-arginine between carboxy and amino groups during the immobilization reaction. A schematic representation of the procedure for conjugating L-AME to FG-beads is depicted in Fig. 3. Briefly, epoxy groups on FG beads were aminolysed by NH_4OH and coupled to EGDE to produce FGNEGDE beads. EGDE, introduced as a spacer is important for the reduction of steric hindrance. Epoxy groups on FGNEGDE beads were aminolysed by NH_4OH, carboxylated with succinic anhydride and activated with NHS to produce NHS-activated FGNEGDENS beads. L-AME was then conjugated to NHS-activated FGNEGDENS. We selected NHS-activated FGNEGDENS beads as a carrier, because L-AME-immobilized beads prepared in this strategy exhibited the minimal non-specific binding of proteins to the beads due to the neutrality of amide groups derived from the coupling reaction between the amino groups of L-AME and the NHS-activated carboxy groups of the beads (data not shown). In addition, the coupling reaction between NHS-activated FGNEGDENS beads and L-AME enabled us to determine the amount of immobilized L-AME.

Evaluation of L-AME-immobilized Beads (Fig. 4)

L-AME-immobilized beads were analyzed for their binding ability to some AIFs such as iNOS (Fig. 4) and non-AIFs such as PPARγ. These radiolabeled proteins were mixed with L-AME-immobilized beads (AL) or control beads (L), and bound proteins were analyzed using SDS-PAGE. iNOS bound to L-AME-immobilized beads and not to control beads. The recovery rate of iNOS from L-AME-immobilized beads was 9.6%, which is a reasonable value for affinity purification because the interaction between iNOS and L-AME is transient. PPARγ bound neither to L-AME-immobilized beads nor to control beads. These results suggest that AIFs can be purified and non-AIFs cannot be purified in this system.

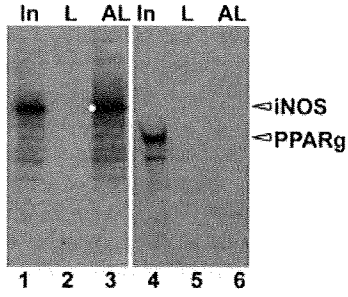

Figure 4. Evaluation of L-AME-immobilized beads. *In vitro* radiolabeled iNOS and PPARγ were mixed with L-AME-immobilized beads (AL) or control beads (L), and bound proteins were recovered. Five percent of input (In), the eluate fraction from control beads (L) and the eluate fraction from L-AME-immobilized beads (AL) were analyzed using SDS-PAGE (5–20% gradient gel). The open circle indicates the bound protein.

Purification and Identification of AIFs by L-AME-immobilized Beads (Figs 5 and 6)

Instead of recombinant proteins, crude cytoplasmic extract of HeLa cells was mixed with L-AME-immobilized beads to purify unknown AIFs from HeLa cells. The bound proteins were separated by SDS-PAGE and visualized by silver staining. The patterns of bound proteins were almost same in the four independent purifications (data not shown). All of the bound proteins obtained from the four purifications were put together and subjected to SDS-PAGE/silver staining analysis (Fig. 5A). Among the specifically bound proteins, three polypeptides of 80, 51 and 50 kDa were successfully identified respectively as phosphofructokinase (PFK), RuvB-like 2 (RBL2), and RuvB-like 1 (RBL1) by mass spectrometry analysis (Fig. 5B, C). We did not proceed to analysis of other bound proteins that were identified as predicted or hypothetical proteins.

The radiolabeled recombinant proteins of PFK, RBL1 and RBL2 were prepared in order to confirm the L-arginine binding activity of these proteins. These radiolabeled proteins were mixed with L-AME-immobilized beads (AL) or control beads (L), and bound proteins were analyzed using SDS-PAGE (Fig. 6A). These three proteins bound to L-AME-immobilized beads and not to control beads. The recovery rates of PFK, RBL1 and RBL2 from L-AME-immobilized beads were 2.2, 1.4 and 1.2% respectively. These recovery rates suggested that the binding activities of PFK, RBL1

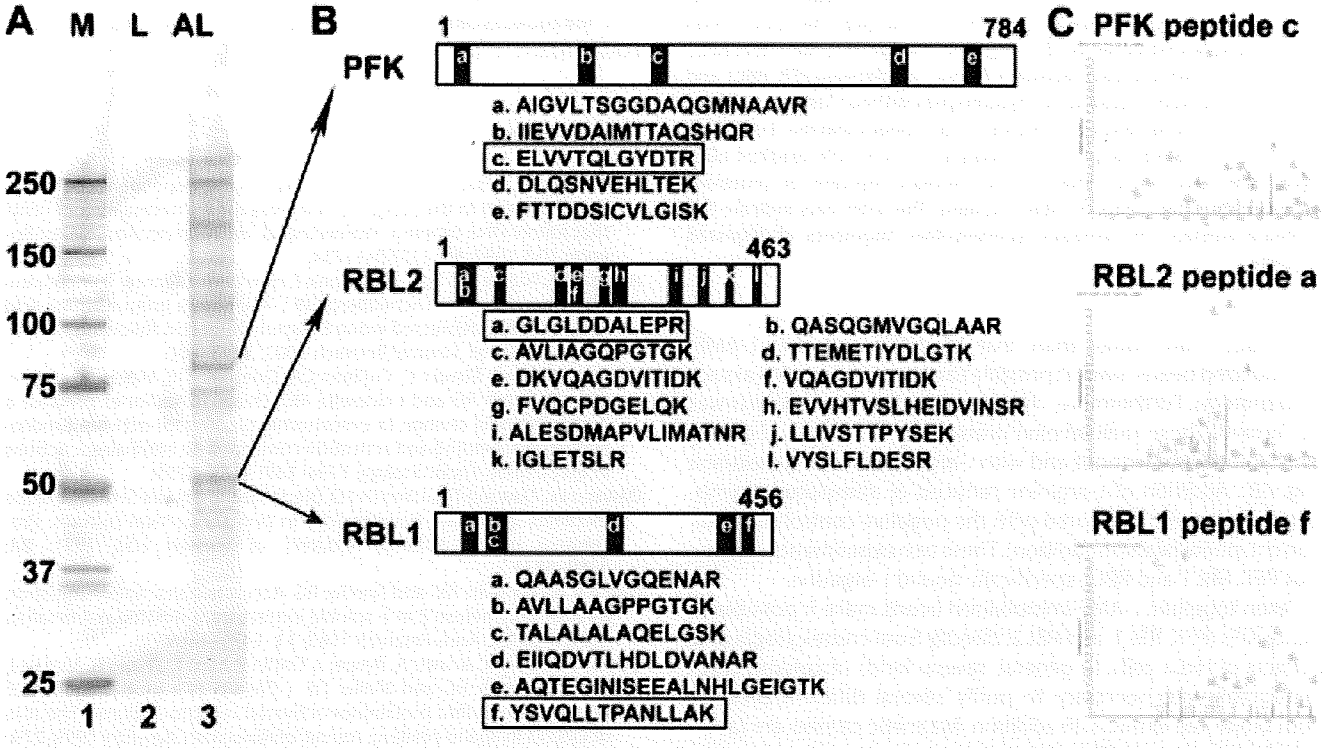

Figure 5. Purification and identification of AIFs from HeLa cell extracts by L-AME-immobilized beads. (A) Purification of AIFs. HeLa cell extracts were mixed with L-AME-immobilized beads (AL) or control beads (L), and bound proteins were separated by SDS-PAGE (5–20% gradient gel) and visualized by silver staining. (B) Identification of AIFs. Three polypeptides were identified by ion-spray mass spectrometry. The identified amino acid sequences are indicated. (C) MS/MS spectra of tryptic peptides of PFK, RBL1 and RBL2. The results of tryptic peptides from PFK/peptide-c, RBL2/peptide-f and RBL1/peptide-a are indicated. Fragment ions B have a charge on the N-terminal, and fragment ions Y have a charge on the C-terminal. Fragment ions B* and Y* are ions that have lost ammonia, and B0 and Y0 are ions that have lost water.

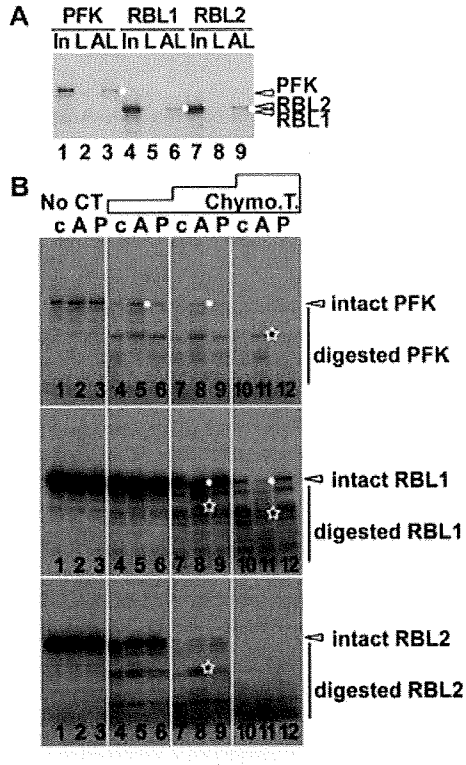

Figure 6. Evaluation of L-arginine binding activity of newly identified AIFs. (A) *In vitro* radiolabeled PFK, RBL1 and RBL2 were mixed with L-AME-immobilized beads (AL) or control beads (L), and bound proteins were recovered. Five percent of input (In), the eluate fraction from control beads (L) and the eluate fraction from L-AME-immobilized beads (AL) were analyzed using SDS-PAGE (5–20% gradient gel). The open circles indicate the bound proteins. (B) L-Arginine change PFK, RBL1 and RBL2 sensitivity to chymotrypsin digestion. *In vitro* radiolabeled PFK, RBL1 and RBL2 were partially digested by chymotrypsin without (lanes 1, 4, 7 and 10), with L-arginine (lanes 2, 5, 8 and 11) or L-phenylalanine (lanes 3, 6, 9 and 12). Digested samples were analyzed in a 5–20% gradient SDS-PAGE. The open circles indicate the protease-resistant or protease-hypersensitive fragments of intact proteins. The solid stars indicate the protease-resistant or protease-hypersensitive fragments of digested proteins.

and RBL2 were lower than that of iNOS, and that L-AME-immobilized beads made it possible to recover the weakly associated proteins. Furthermore, chymotrypsin digestion analysis was performed. These radiolabeled proteins were partially digested by chymotrypsin without and with L-arginine or L-phenylalanine (Fig. 6B). Addition of L-arginine reduced or stimulated chymotrypsin sensitivity compared with the negative control (no addition or L-phenylalanine addition). These two experiments indicate that PFK, RBL1 and RBL2 specifically bound L-arginine.

Taken together, L-AME-immobilized beads make it possible to purify AIFs (PFK, RBL1, and RBL2) directly from crude cytoplasmic extracts of HeLa cells. In general, several kinds of column chromatography are necessary to purify several different proteins from crude cell extracts. In addition, Magnetic carriers are favorable for automated high-throughput screening systems using permanent magnet. The recently developed magnetic carriers used in this study have been shown to have higher performance compared with commercially available magnetic (Nishio *et al.*, 2008). Therefore, L-AME-immobilized beads developed in this study are useful for efficient purification of AIFs from various types of cells, from widely different conditions, or in the presence of a variety of reagents.

Conclusions

L-Arginine, a semi-essential amino acid in humans, is one of the most metabolically versatile amino acids and serves as a precursor for the synthesis of urea, nitric oxide, polyamines, proline, glutamate, creatine and agmatine. L-Arginine is metabolized through a complex and highly regulated set of pathways and has multiple functions that remain incompletely understood.

In order to purify, identify and study AIFs, we have newly developed L-AME-immobilized beads. In this study, we showed that this system is very effective for purification of AIFs directly from crude cell extracts. We conclude that L-AME-immobilized beads are powerful tool for purification of AIFs. We expect that L-AME-immobilized beads will contribute to the purification of AIFs from different kinds of cells such as pancreatic beta cells, pituitary cells and endothelial cells. The identification and characterization of these AIFs should reveal the mechanism of various biological function of L-arginine, such as stimulation of glucose-induced insulin secretion, induction of growth hormone release, and endothelial cell function.

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