FR225659-binding Proteins: Identification as Serine/Threonine Protein Phosphatase PP1 and PP2A Using High-performance Affinity Beads

HIDETAKA HATORIa,*, TATSUYA ZENKOHb, MOTOO KOHAYASHIA, YOSHIHIRO OHTSUa, NOBUHARU SHIGEMATSA, HIROYUKI SETOIB, MOTOHIRO HINOC and HIROSHI HANDAe

a Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-2698, Japan
b Medicinal Chemistry Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 2-1-6 Kashima, Yodogawa, Osaka 532-8514, Japan
c Fermentation Development Laboratories, Fujisawa Pharmaceutical Co., Ltd., 156 Shinkawa, Nishikasugai, Aichi 452-0915, Japan
d Graduate school of Bioscience and Biotechnology and e Frontier Collaborative Research Center, Tokyo Institute of Technology, Yokohama 226-8503, Japan

(Received for publication January 13, 2004)

FR225659 was originally isolated as a novel gluconeogenesis inhibitor produced by fungal strain Helicomyces sp. No. 19353. To identify the target protein of FR225659, we synthesized high-performance affinity latex beads that immobilized FR225659 derivative FR253761 or FR259383. Using these beads, we identified FR225659 binding proteins as serine/threonine protein phosphatase type1 (PP1) and type2A (PP2A) from rat hepatocyte crude extract. FR225659 and its synthetic derivatives were strongly inhibited the enzyme activities of purified catalytic subunits of PP1 and PP2A in vitro.

To date, a number of naturally occurring pharmacologically active compounds have been screened and discovered without elucidation of their target molecules or precise pharmacological mechanisms. Despite their unique biological activities, many of these compounds were not used for clinical purposes, because of their undesirable side effects. If the target molecules of these natural products are identified, it may be possible that the more potent or less toxic compound is discovered from the structure based rational screening or drug design.

Although affinity purification is a well-established technique as a powerful method of ligand binding proteins, widespread use of the technique has been limited by the poor characteristics of commercially available matrices. We have previously developed the latex beads which consist of polystyrene and polyglycidyl methacrylate copolymer that were originally used for the affinity purification of DNA-binding proteins1-3). The hydrophilic polyglycidyl surface shows relatively few non-specific adsorptions of proteins and can be reacted with amino group to load ligands. Furthermore, the large total surface area is especially effective in purifying the receptor from a small amount of cell lysate. We have recently reported that receptor purification of a small molecules such as E-3330 and FK506 using this latex particles4).

FR225659 (1) was originally isolated as potent gluconeogenesis inhibitor produced fungal strain Helicomyces sp. No. 193535). This compound possesses a very unique structure that consists of a novel acyl-group and three unusual amino acids6). 1 inhibits glucagon-stimulated gluconeogenesis in primary rat hepatocytes in vitro, and shows hypoglycemic activity in two different in vivo models7). This compound does not inhibit two major rate limiting enzymes of gluconeogenesis pathway, i.e. glucose-6-phosphatase and fructose-1,6-bisphosphatase. 1 also does not interfere the binding of glucagon and its cell surface receptor. So the target molecule of 1 is still unclear.

For the aim of target molecule identification of 1, we

* Corresponding author: hidetaka_hatori@po.fujisawa.co.jp
synthesized bioactive derivatives of 1, FR253761 (2), FR259383 (3) and FR259384 (4) (Fig. 1), and prepared high-performance affinity beads that immobilized 2 or 3 previously. Using these beads we screened binding proteins of 1 from rat hepatocyte crude extract. We describe here, identification of 1 binding proteins as serine/threonine protein phosphatase type 1 (PP1) and type 2A (PP2A), and direct inhibition of PP1 and PP2A enzyme activity by 1 and its synthetic derivatives.

Materials and Methods

Preparation of Rat Hepatocyte Extracts

Rat hepatocytes were isolated by the collagenase perfusion method for 24 hour fasted male Wister rats (200–220 g). Nuclear extract and cytoplasmic fraction of hepatocytes were prepared as described.

In Vitro Gluconeogenesis Assay

Measurement of in vitro gluconeogenesis activities of isolated rat hepatocyte was described previously.

Preparation of Drug Immobilized Matrices

Latex beads for affinity purification (SG beads) was prepared as described previously. Chemical synthesis of compound 2, 3 and 4, plus preparation of latex beads which immobilized 2 or 3 were previously described. The amount of immobilization of 2 or 3 on latex beads were 38.9 nmol/mg or 15.0 nmol/mg, respectively.

Affinity Purification of Drug Binding Proteins

0.1 mg of latex beads which immobilized 2 or 3 were equilibrated with buffer E (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 0.2 mM EDTA, 10% Glycerol, 0.1% Nonidet P-40, 1 mM CaCl₂, 0.5 mM DTT), mixed with 200 µl of cytoplasmic fraction (0.45 mg protein) or nuclear extract (0.2 mg protein) of rat hepatocytes and incubated at 4°C for one hour with occasional agitation. After washing buffer E, binding proteins were eluted with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and subjected to silver staining. For competition analysis, cytoplasmic fraction or nuclear extract was pre-incubated with indicated concentration of 1 or 2 for one hour at 4°C with occasional agitation followed by affinity purified.

To estimate the binding proteins, protein bands that competes free ligands were digested with trypsin, followed by peptide-mass fingerprinting was performed using metrics-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

Immunoblotting

Immunoblotting was performed as described using anti-PP1Cα, anti-PP1Cβ, anti-PP1Cγ, anti-PP2A catalytic subunit and anti-PP2A regulatory subunit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Protein Phosphatase Assay

Purified catalytic subunits of PP1 and PP2A were purchased from Upstate Biotechnology, Inc (Lake Placid, NY). Okadaic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Protein phosphatase activities were
measured using 10 μM of 32P-labelled glycogen phosphorylase α as substrate and assayed according to previously described procedure12).

Results

Inhibition of Hepatic Gluconeogenesis

The abilities of 1, 2, 3 and 4 to inhibit glucagon-stimulated gluconeogenesis in primary rat hepatocytes in vitro are summarized in Table 1. The 50% inhibitory concentration (IC50) of 1 was 0.19 μM. Compound 2, 3 or 4 also possess inhibitory activities with IC50 values 0.83 μM, 1.3 μM or 6.0 μM, respectively. This result indicates that synthetic derivatives could also bind to the target molecule of 1 in similar concentration.

Affinity Purification of Drug Binding Proteins

Fig. 2A shows the existence three peptides which specifically binds to 2 immobilized latex beads and these binding are dose dependently competed by the addition of free 1 in rat hepatocyte cytoplasmic fraction (indicated solid arrows). Presumed molecular weight of these peptides are 36 kDa, 37 kDa, and 60 kDa. As the result of peptide mass finger printing, we estimated these proteins were PP1 catalytic subunits (PP1Cs), PP2A catalytic subunit and PP2A regulatory subunit (Fig 2B).

Immunoblot Analysis

Immunoblot analysis indicated that these peptides which bind to drug immobilized affinity beads were PP1Cα, PP1Cβ, PP1Cγ, PP2A regulatory subunit and PP2A catalytic subunit in rat hepatocyte cytoplasmic fraction (Fig. 3 lane 2). In nuclear fraction, we identified PP1Cα,

Table 1. Inhibitory effects of 1, 2, 3 and 4 on glucagon induced gluconeogenesis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition of hepatocyte gluconeogenesis (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.52</td>
</tr>
<tr>
<td>2</td>
<td>0.83</td>
</tr>
<tr>
<td>3</td>
<td>1.3</td>
</tr>
<tr>
<td>4</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Fig. 2. Affinity purification and estimation of 1 binding protein.

(A) Affinity purification and competition analysis of 1 binding proteins. A 200 μl samples of rat hepatocyte cytoplasmic fraction was incubated in the presence (lanes 3–6) or absence (lanes 1, 2) of 1. Then 0.1 mg of 2 immobilized beads (lanes 2–6) or beads alone (lane 1) were added. After incubation for 1 hour at 4°C, the beads were washed and analyzed 12.5% SDS-PAGE followed by silver staining.

(B) Estimation of 1 binding proteins as serine/threonine protein phosphatase type1 catalytic subunits (PP1Cs), type2A (PP2A) catalytic subunit and PP2A regulatory subunit by peptide-mass fingerprinting.
PP1Cβ, PP1Cγ as binding proteins (Fig. 3 lane 6). These proteins bind to the latex by drug immobilization specific manner, this binding were inhibited by existence of 10 μg/ml of free 1 (Fig. 3 lanes 3, 7) or 2 (Fig. 3 lanes 4, 8).

Inhibition of Protein Phosphatase Activity

IC₅₀ values and PP2A/PP1 ratio of IC₅₀ of compounds 1 to 4 and Okadaic acid are summarized in Table 2. All compounds potently inhibited phosphatase activities of PP1 and PP2A.

Discussion

In this paper, we describe the affinity purification and identification of naturally occurring gluconeogenesis inhibitor 1 binding proteins as PP1 and PP2A, and direct inhibition of PP1 and PP2A enzyme activity by 1 and its synthetic derivatives.

For the purpose of searching for 1 binding protein using affinity beads technology, we previously designed and synthesized biologically active derivatives which have suitable amine moiety and stable structure for immobilization to the matrices⁹. As shown in Fig. 1, compound 2, 3 and 4 have a primary amine group, which is used for immobilizing to matrices, at the end of linker attached to phenolic hydroxyl group in carboxy-terminal acyl-group. 3-chrolo-4-hydroxy arginine residue originally exists in 1 is substituted for arginine or lysine residue because of improvement of their chemical stability. Because of 3 which has two primary amine group (ε-amino group of lysine residue and amine spacer attached to carboxy-terminal phenolic hydroxyl group), we prepared 4 immobilized latex followed by deprotection of Boc group to prepare 3 immobilized latex⁹. All synthetic compounds possess inhibitory activities against glucagon induced gluconeogenesis in vitro (Table. 1). Although in vitro activities of 2, 3 or 4 are less effective than 1, we think it would be enough to identify their binding proteins.

Using the latex beads immobilized 2, we screened binding proteins from crude rat liver extract. From cytoplasmic fraction, we detected three peptides which bind specifically 2 immobilized latex. Their binding to the latex is competed with free compound 1 by concentration dependent manner (Fig. 2A indicated solid arrows). The
same result was obtained that compound 2 was used for competition. As results of peptide-mass finger printing, we estimated these proteins were PP1Cα, PP2A catalytic subunit and PP2A regulatory subunit (Fig. 2B). On the other hand, from rat liver nuclear extract, only putative PP1C peptides seems to bind to affinity beads (data not shown). When used the affinity latex immobilized 3, we detected essentially the same peptides in cytoplasmic fraction and nuclear extract which compete free 1 or 3 by concentrate dependent manner (data not shown).

Immunoblot analysis used specific antibodies for PP1C subtypes and PP2A subunits indicated that the peptides which binding to drug immobilized affinity beads were PP1Cα, PP1Cβ, PP1Cγ, PP2A regulatory subunit and PP2A catalytic subunit in cytoplasmic fraction (Fig. 3 lane 2). Whilst in nuclear fraction, we identified PP1Cα, PP1Cβ, PP1Cγ as binding proteins (Fig. 3 lane 6). These proteins were bound to the latex beads by drug immobilization dependent manner and their binding was inhibited by the addition of free compound 1 or 2 (10 μg/ml). When we used 10 μg/ml of compound 2 as a competitor, binding of PP2A catalytic and regulatory subunits to drug immobilized latex was not completely inhibited while same concentration of 1 completely inhibited (Fig. 3 lane 3, 4). This result indicates binding affinity to PP2A subunits of 2 is much weaker than 1, and is parallel to the IC\textsubscript{50} values of PP2A inhibition of these compounds (Table 2). Similar results were obtained when using 3 immobilized latex beads (data not shown).

To clarify that compound 1, and its synthetic analogues affect to enzymatic activities of PP1 or PP2A catalytic subunits, we assayed inhibitory effects of phosphatase activity purified PP1 or PP2A catalytic subunits. As indicated in Table 2, all compounds strongly inhibited protein phosphatase activities of PP1 and PP2A when \textsuperscript{32}P-labelled glycogen phosphorylase a as substrate. According to the ratio of IC\textsubscript{50} value of PP1 and PP2A, compound 1 to 4 are more specific inhibitor to PP1 than PP2A. Especially, synthetic derivatives 2, 3 and 4 are extremely specific to PP1 with the ratio of IC\textsubscript{50} ranged 600 to 1500. In comparison with gluconeogenesis inhibitory activity summarized in Table 1, specific inhibition of PP1 may be more critical for inhibition of hepatic gluconeogenesis. Indeed PP2A specific inhibitor Okadaic acid did not inhibit hepatic gluconeogenesis at non-cytotoxic concentration, and synthetic derivatives of 1 which did not have inhibitory activity of gluconeogenesis are also non-potential against PP1 activity (data not shown).

Protein phosphatases regulate many cellular functions and signal transduction pathways in cooperation with protein kinases\textsuperscript{13,14}. PP1 and PP2A are two of four major protein serine/threonine phosphatases that regulate diverse cellular events such as cell division, transcription, translation, muscle contraction, glycogen metabolism, and neuronal signaling\textsuperscript{15}. Because of PP1 catalytic subunits are encoded by three different genes and associated with a number of targeting subunits, for the investigation of detailed cellular functions of PP1, it is useful that usage of selective inhibitors of these enzymes.

To date, a number of naturally occurring structurally diverse families of inhibitors which inhibit PP1 as well as PP2A, have been identified and purified. Most of these compounds inhibit PP1 and PP2A activity at same potency, with the exception of compounds such as Okadaic acid, Thysrsiferyl 23-acetate (TF-23A), foscirecin, and tautomyecetin. Okadaic acid, TF-23A and foscirecin selectively inhibit PP2A activity\textsuperscript{16-18}, and their selectivity has made it possible to analyze PP2A function in living cells. Tautomyecetin is recently reported PP1 selective inhibitor. The PP1/PP2A ratio of IC\textsubscript{50} of tautomyecetin was reported to be within range of 23 to 46\textsuperscript{19}. 1 and its derivatives are structurally unrelated to all reported PP1/PP2A inhibitors and possess extreme specificity to PP1 with the ratio of IC\textsubscript{50} ranged from 20 to 1500. Therefore 1 and its derivatives maybe useful to clarify the roles of protein phosphatases especially PP1 in cellular functions including glucose metabolism in liver, and to develop a novel gluconeogenesis inhibitor which improved potency and safety than 1.

Acknowledgement

The authors were grateful to Ms. TERUMI OKUDAIRA for the assistance of in vitro gluconeogenesis assay. This work was performed under the management of the Research Association.
for Biotechnology as a part of the Industrial Science and Technology Frontier Program supported by NEDO (New Energy and Industrial Technology Development Organization).

References


