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Purification and Identification of Activating Enzymes of CS-0777, a Selective Sphingosine 1-Phosphate Receptor 1 Modulator, in Erythrocytes*[S]

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CS-0777 is a selective sphingosine 1-phosphate (S1P) receptor 1 modulator with potential benefits in the treatment of autoimmune diseases, including multiple sclerosis. CS-0777 is a prodrug that requires phosphorylation to an active S1P analog, similar to the first-in-class S1P receptor modulator FTY720 (fingolimod). We sought to identify the kinase(s) involved in phosphorylation of CS-0777, anticipating sphingosine kinase (SPHK) 1 or 2 as likely candidates. Unlike kinase activity for FTY720, which is found predominantly in platelets, CS-0777 kinase activity was found mainly in red blood cells (RBCs). *N,N*-Dimethylsphingosine, an inhibitor of SPHK1 and -2, did not inhibit CS-0777 kinase activity. We purified CS-0777 kinase activity from human RBCs by more than 10,000-fold using ammonium sulfate precipitation and successive chromatography steps, and we identified fructosamine 3-kinase (FN3K) and fructosamine 3-kinase-related protein (FN3K-RP) by mass spectrometry. Incubation of human RBC lysates with 1-deoxy-1-morpholinofructose, a competitive inhibitor of FN3K, inhibited ~10% of the kinase activity, suggesting FN3K-RP is the principal kinase responsible for activation of CS-0777 in blood. Lysates from HEK293 cells overexpressing FN3K or FN3K-RP resulted in phosphorylation of CS-0777 and structurally related molecules but showed little kinase activity for FTY720 and no kinase activity for sphingosine. Substrate preference was highly correlated among FN3K, FN3K-RP, and rat RBC lysates. FN3K and FN3K-RP are known to phosphorylate sugar moieties on glycosylated proteins, but this is the first report that these enzymes can phosphorylate hydrophobic xenobiotics. Identification of the kinases responsible for CS-0777 activation will permit a better understanding of the pharmacokinetics and pharmacodynamics of this promising new drug.

Multiple sclerosis is an inflammatory disorder of the brain and spinal cord in which focal lymphocytic infiltration leads to damage of myelin and axons (1). Treatment of multiple sclerosis has primarily depended upon immunomodulatory drugs, such as IFN-β, glatiramer acetate, and natalizumab. However, these drugs are only partially effective and/or have problematic side effects (1–3), and there is a clear unmet need for improved treatments for multiple sclerosis. Sphingosine 1-phosphate (S1P)[4] receptor modulators are emerging as a new class of drugs with potential therapeutic application in multiple sclerosis (4, 5).

Sphingosine is phosphorylated to S1P by sphingosine kinase 1 (SPHK1) and sphingosine kinase 2 (SPHK2) in humans (6–9). S1P (supplemental Fig. 1) is a bioactive signaling molecule that activates a family of G-protein-coupled receptors, known as $S1P_1$–$S1P_5$ (4). Among these five S1P receptors, $S1P_1$ is a major regulator of egress of mature T cells from the thymus and of T and B cells from lymph nodes into blood and lymph (10–12).

Discovery of FTY720 (fingolimod, chemical structure in Fig. 1), the first-in-class S1P receptor modulator, has opened a new field of S1P receptor biology and contributed to an improved understanding of the regulation of immune function (13–15). FTY720 is a prodrug, which, like sphingosine, requires phosphorylation *in vivo* for its biological activity. Orally administrated FTY720 is phosphorylated predominantly by SPHK2 to an S1P analog (FTY720 phosphate; Fig. 1) that induces internalization of $S1P_1$ on lymphocytes, thereby blocking the ability of the receptor to support lymphocyte egress and recirculation through secondary lymphoid organs. This suppresses immune responses and presumably serves as the main immunomodulatory mechanism of FTY720 in animals and humans (10, 16–20). Two phase III studies of FTY720 demonstrated superior clinical efficacy to placebo and IFN-β in patients with relapsing-remitting multiple sclerosis (21, 22), and FTY720 was recently approved by

* All of the authors are employees of Daiichi Sankyo Co., Ltd., which is developing CS-0777.

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[4] The abbreviations used are: S1P, sphingosine 1-phosphate; DMF, 1-deoxy-1-morpholinofructose; DMS, *N,N*-dimethylsphingosine; FN3K, fructosamine 3-kinase; FN3K-RP, fructosamine 3-kinase-related protein; HIC, hydrophobic interaction chromatography; LC-MS/MS, liquid chromatography equipped with tandem mass spectrometry; $S1P_1$, sphingosine 1-phosphate receptor 1; $S1P_3$, sphingosine 1-phosphate receptor 3; SPHK1, sphingosine kinase 1; SPHK2, sphingosine kinase 2; Bis-tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

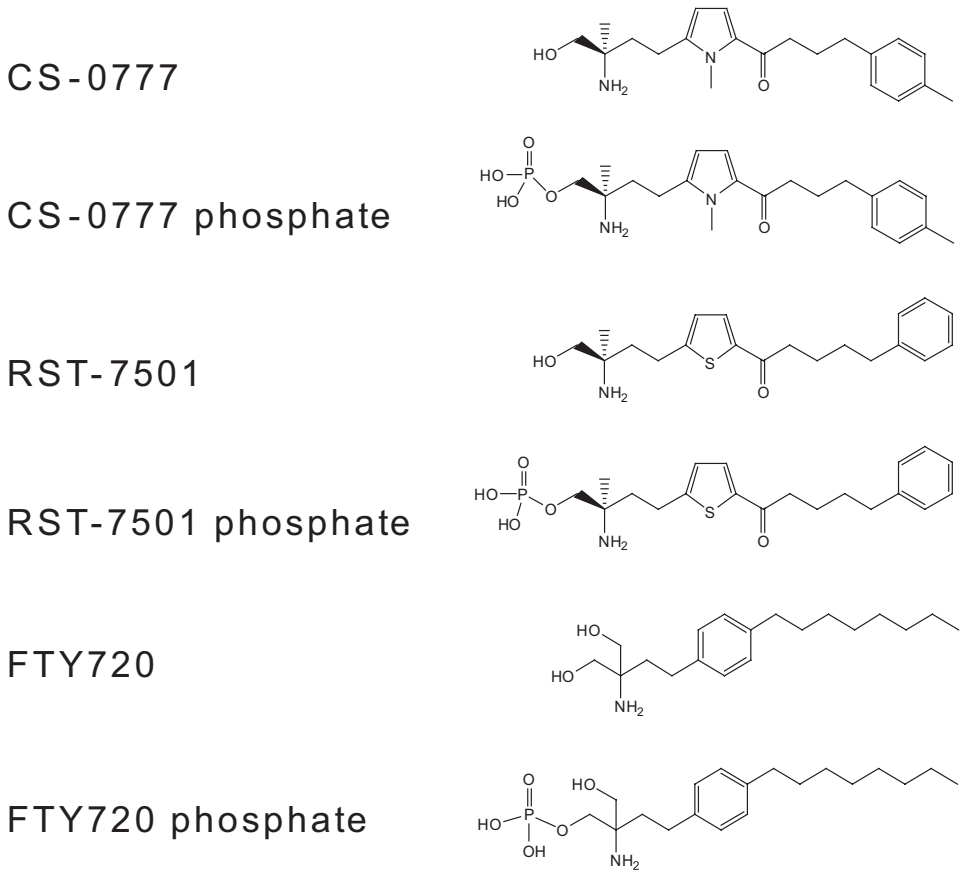

FIGURE 1. **Chemical structures of CS-0777, RST-7501, FTY720, and their phosphorylated metabolites.**

United States Food and Drug Administration as a first-line treatment for relapsing forms of multiple sclerosis.

CS-0777 (Fig. 1) is a novel selective $S1P_1$ modulator in early development for autoimmune diseases, including multiple sclerosis (24). Like FTY720, CS-0777 is a prodrug, phosphorylated *in vivo* to an active S1P analog (Fig. 1), but whereas FTY720-phosphate lacks receptor subtype specificity, having similar potencies for $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ (16), the phosphorylated form of CS-0777 is more selective, with 300-fold greater potency for $S1P_1$ *versus* $S1P_3$ and essentially no activity on $S1P_2$ (24). Although the clinical importance of $S1P_1$ selectivity is still unknown, off-target effects mediated through S1P receptors may contribute to side effects of FTY720 observed in clinical trials (13). For example, initial studies in rodents led to the hypothesis that the bradycardia observed with FTY720 was mediated through $S1P_3$ (25, 26); however, findings of dose-related bradycardia with other $S1P_1$-selective modulators has led to uncertainty about the importance of $S1P_1$ selectivity to bradycardia in humans (13).

CS-0777 demonstrated immunosuppressive activity in mouse and rat models of experimental autoimmune encephalitis, an established model of multiple sclerosis and autoimmune disease (24). In healthy volunteers, single oral doses of CS-0777 caused pronounced dose-dependent lymphopenia, including reversible decreases in circulating T and B cells (27). Furthermore, in multiple sclerosis patients, oral doses of CS-0777 caused a dose-dependent decrease in circulating lymphocytes, with a slightly greater suppression of $CD4^+$ *versus* $CD8^+$ T cells and relative sparing of $CD8^+$ effector memory cells. Therefore, CS-0777 is anticipated to demonstrate immunomodulatory activity through selective $S1P_1$ modulation, similar to FTY720, without engaging other S1P receptors, which may have benefits in circumventing off-target toxicities.

Identification of the mechanisms of metabolic activation is critical for understanding the pharmacological (pharmacokinetic and pharmacodynamic) properties of prodrugs. Orally administrated CS-0777 is rapidly phosphorylated and reaches equilibrium with CS-0777-phosphate in blood as in the case of FTY720 (16), suggesting that, in blood, phosphorylation is balanced by de-phosphorylation by phosphatases. Unlike FTY720 and FTY720-phosphate, however, the equilibrium for CS-0777 and CS-0777-phosphate strongly favors the phosphorylated form (27). Because SPHK1 and SPHK2 are the principal kinases involved in sphingosine phosphorylation, and SPHK2 is reported to be the major kinase responsible for FTY720 phosphorylation (17, 28, 29), we speculated that SPHK1 or SPHK2 may be involved in the phosphorylation of CS-0777. Surprisingly, however, we identified two unrelated kinases, fructosamine 3-kinase (FN3K) and FN3K-related protein (FN3K-RP), as the principal enzymes responsible for CS-0777 activation in blood. We report here the identification of FN3K and FN3K-RP as CS-0777 kinases and explore the kinase substrate specificity for a number of related compounds identified in the search for a selective $S1P_1$ modulator.

EXPERIMENTAL PROCEDURES

Chemicals—All chemical compounds were synthesized in Daiichi Sankyo Co., Ltd. Chemical structures are illustrated in Fig. 1 and supplemental Fig. 1.

Preparation of Human RBC Lysates—Human blood specimens were obtained from healthy volunteers according to a protocol approved by the Research Ethics Committee at Daiichi Sankyo Co., Ltd. All procedures below were conducted at 4 °C. Equal amounts (100 ml) of blood from five subjects were combined and mixed, and 3.2% (w/v) of sodium citrate was added as an anticoagulant. Blood was centrifuged at 1,600 × *g* for 5 min, and the precipitated RBCs were collected. RBCs were washed four times with 20 mM HEPES, pH 7.4, containing 138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, and 1 mg/ml glucose and hemolyzed with 20 mM HEPES, pH 7.4, containing 3.3 mM NaH_2PO_4, 2.9 mM KCl, 5 mM $MgCl_2$, 1 mg/ml glucose, 1 mM DTT, and a protease inhibitor mixture (complete EDTA-free, Roche Applied Science). The hemolysate was centrifuged at 100,000 × *g* for 1 h, and the supernatant (designated human RBC lysate) was used for subsequent experiments. The lysate was aliquoted, quickly frozen by liquid nitrogen, and stored at −80 °C until use. Rat RBC was prepared using similar methods.

Assay for Phosphorylation of CS-0777 and Related Compounds—Whole blood, RBCs, or RBC lysate samples were incubated at 37 °C for 3 h with CS-0777 or related compounds in 5 mM $MgCl_2$, 1 mM DTT, 5 mM ATP, and 0.5% CHAPS in 250 μl of 100 mM HEPES, pH 7.4. The concentrations of test compounds are provided for each experiment in the figure legends. The sample was mixed with 500 μl of methanol to stop the reaction and centrifuged, and 20 μl of the supernatant was subjected to reversed-phase chromatography (YMC-pack ODS A-312 column, 6.0 mm internal diameter × 150 mm length) at 40 °C. The substrate and product were separated with a linear gradient elution of acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. The gradient slope was modified depending on the compound studied. Absorbance at 230, 254, or 295 nm was monitored, and phosphorylation of CS-0777 and related compounds was determined by measuring the peak area of the phosphorylated compound. For FTY720 and sphingosine, which lack UV absorbance, the phosphorylated product was quantitated by liquid chromatography equipped with tandem mass spectrometry (LC-MS/MS) (API4000, Applied Biosystems).

RST-7501 Kinase Assay for Protein Purification—RST-7501, a closely related analog of CS-0777 (Fig. 1), was selected for the purification of the CS-0777-related kinase because of the high level of UV absorbance of phosphorylated RST-7501 and hence its amenability to easy detection during fractionation and purification steps. Samples were incubated at 37 °C for 1 h with 100 μg/ml RST-7501, 1 mM ATP, and 0.5% CHAPS in 75 μl of 100 mM HEPES, pH 7.0. In some experiments, 1-deoxy-1-morpholinofructose (DMF) (Sigma) was added at a final concentration of 1 mM to inhibit FN3K activity. To stop the reaction, the sample was mixed with 150 μl of methanol, and 10 μl of the filtrate (0.45 μm, Millipore) was subjected to reversed-phase chromatography (TSK-gel ODS-100S column, 4.6 mm internal diameter × 150 mm length) at 40 °C. The substrate and product were separated by isocratic elution with 40% acetonitrile containing 0.1% TFA at a flow rate of 1 ml/min. Absorbance at 295 nm was monitored, and RST-7501 kinase activity was determined by measuring the peak area of RST-7501-phosphate. One unit of RST-7501 kinase activity was defined as the activity required for 1 μg/ml production of RST-7501-phosphate under these experimental conditions.

Purification of FN3K and FN3K-RP—All purification procedures were conducted at 4 °C. Human RBC lysate was mixed with ammonium sulfate to reach 10% saturation, stirred for 3 h, and centrifuged at 6,700 × *g* for 30 min. The resultant supernatant was further mixed with ammonium sulfate to reach 50% saturation, stirred for 3 h, and then centrifuged at 12,000 × *g* for 30 min. The precipitate from the second centrifugation (designated 10–50% saturated ammonium sulfate precipitate) was collected and stored at −20 °C until use.

For FN3K, a sample of 10–50% saturated ammonium sulfate precipitate equivalent to 75 ml of human RBC lysate was dissolved in 100 ml of 20 mM HEPES, pH 7.0, containing 1 mM DTT and 5 mM $MgCl_2$ (HMD buffer) and dialyzed against 5 liters of HMD buffer containing 2 M NaCl. The dialyzed sample was centrifuged at 12,000 × *g* for 15 min, and 5–10 ml of the supernatant was filtered and loaded onto a hydrophobic interaction chromatography (HIC) column (5 ml of HiTrap phenyl-HP, GE Healthcare). Bound proteins were eluted using a linear gradient from 2 to 0 M NaCl in HMD buffer, and samples from each fraction were tested for RST-7501 kinase activity. The active fractions were combined (total volume, 60 ml) and dialyzed against 2 liters of HMD buffer containing 0.5% CHAPS (HMDC buffer). The sample was then loaded onto a dye-affinity column (two columns linearly connected, HiTrap Blue HP, 1 ml, GE Healthcare). Bound proteins were eluted using a linear gradient of 0–2 M NaCl in HMDC buffer. Active fractions were combined (4 ml), dialyzed against 500 ml of HMDC buffer, and then loaded onto an anion-exchange column (1 ml of Resource Q, GE Healthcare). The effluent from the Resource Q column was then loaded onto a cation-exchange column (1 ml of Resource S, GE Healthcare), and proteins were eluted using a linear gradient of 0–2 M NaCl in HMDC buffer. Active fractions (4 ml) were dialyzed against 500 ml of HMDC buffer and loaded onto a high resolution cation-exchange column (Mono S PC 1.6/5, GE Healthcare) and proteins were further eluted as above. A portion of the active fraction (50 μl) from the Mono S column was loaded onto a gel filtration column (Superdex 75 PC 3.2/30, GE Healthcare) equilibrated with HMDC buffer containing 150 mM NaCl. Samples (10 μl) from the active and adjacent fractions were subjected to SDS-PAGE (10–20% gradient gel, Bio-Rad) under reducing condition. The gel was stained with a fluorescent dye, SYPRO® Ruby (Molecular Probes) according to the manufacturer's protocol, and band intensity was quantified using a fluorescent scanner, Molecular Imager FX (Bio-Rad) using Quantity One software (Bio-Rad).

For purification of FN3K-RP, a sample of the 10–50% saturated ammonium sulfate precipitate equivalent to 50 ml of human RBC lysate was dissolved in 50 ml of 20 mM HEPES, pH 9.0, containing 1 mM DTT, 5 mM $MgCl_2$, and 0.5% CHAPS (HMDC9 buffer) and dialyzed against 2 liters of HMDC9 buffer. The dialyzed sample was centrifuged at 12,000 × *g* for 10

min, and the supernatant was loaded onto an anion-exchange column (HiPrep Q 16/10 XL, GE Healthcare). Bound proteins were eluted using a linear gradient of 0–1 M NaCl in HMDC9 buffer. Eluted fractions were tested for RST-7501 kinase activity with or without addition of 1 mM of DMF. DMF-insensitive active fractions were combined (total volume of 10 ml), dialyzed against 500 ml of HMDC buffer, and loaded onto a dye-affinity column (1 ml, HiTrap Blue HP, GE Healthcare). Bound proteins were eluted with HMDC buffer containing 1 M NaCl. Active fractions (total, 1.5 ml) were dialyzed against 500 ml of 20 mM Tris/Bis-tris propane, pH 6.0, containing 1 mM DTT, 5 mM $MgCl_2$, and 0.5% CHAPS (TMDC6 buffer) and loaded onto the high resolution cation-exchange column (Mono S PC 1.6/5). A portion of the active fraction (50 μl) from the Mono S column was loaded onto a gel filtration column (Superdex 75 PC 3.2/30) equilibrated with HMDC buffer containing 150 mM NaCl. Samples (10 μl) from the active and adjacent fractions were subjected to SDS-PAGE, stained with fluorescent dye, and analyzed as above.

Identification of FN3K and FN3K-RP by Mass Spectrometry—To identify the kinases responsible for phosphorylation of CS-0777 and related compounds, bands from the SDS-polyacrylamide gels correlating with kinase activity were excised and destained at 30 °C for 30 min twice with 20 mM NH_4HCO_3, pH 8, containing 50% acetonitrile. The gel pieces were dehydrated with acetonitrile and dried completely using a centrifuge evaporator. Dried gel pieces were rehydrated in 20 mM NH_4HCO_3, pH 8, containing 10 mM DTT and incubated at 50 °C for 30 min to reduce protein disulfides. The gel pieces were then cooled to 20 °C and soaked for 20 min at 20 °C in the dark in 20 mM NH_4HCO_3, pH 8, containing 55 mM iodoacetamide to alkylate free sulfhydryls to prevent further disulfide formation. The gel pieces were further dehydrated with acetonitrile and rehydrated with 20 mM NH_4HCO_3, pH 8, and washed at 20 °C for 15 min. After washing, the gel pieces were dehydrated with acetonitrile and dried completely using a centrifuge evaporator. Dried gel pieces were then rehydrated and incubated for 12 h at 37 °C in 20 mM NH_4HCO_3, pH 8, containing 10 ng/μl trypsin (modified trypsin, Promega) to digest proteins. The resulting peptides were extracted once with formic acid/H_2O buffer (0.05% formic acid) and twice with formic acid/acetonitrile buffer (0.05% formic acid in acetonitrile) for 5 min each. The collected extracts were evaporated to ~20 μl and then analyzed by LC-MS/MS.

LC-MS/MS was carried out using a modified LC-MS/MS system as described previously (30, 31). A Q-TOF2 mass spectrometer (Waters) was equipped with a direct flow nano-liquid chromatography system (Rencon, NanoSolution) (32). A homemade electrospray ionization tip column (150 μm internal diameter × 50 mm length) was packed with reversed-phase C_{18} resin (ODS-HG-3, Nomura Chemical). Elution of peptides was carried out using a linear gradient of acetonitrile in formic acid/H_2O buffer, with a 5-μl sample volume and a flow rate of 100 nl/min. The MS/MS spectra were searched against the GenBank™ nonredundant protein data base compiled by the National Center for Biotechnology Information using the Mascot program (Matrix Sciences).

Protein Assay—Total protein concentrations were determined using a modified Bradford assay (Coomassie Plus Protein Assay, Pierce) with bovine serum albumin as a standard.

Expression of Recombinant Human FN3K-RP and FN3K—A cDNA containing the open reading frame (ORF) of the full-length human FN3K-RP gene was obtained from Invitrogen (clone ID 3351601). The cDNA was subcloned into pcDNA3.1(+) (Invitrogen) between the XhoI and EcoRI sites. The nucleic acid sequence in the expression vector was confirmed by DNA sequencing to be a perfect match with the FN3K-RP sequence submitted to GenBank™ (accession number BC007611).

The cDNA clone for human FN3K was obtained from GeneCopoeia (catalog number GC-W1392). Unexpectedly, DNA sequencing of this cDNA revealed a missense mutation at amino acid 153 (F153S). Therefore, we back-mutated this site using QuickChange multisite-directed mutagenesis kit (Stratagene). The ORF for the full-length human FN3K was subcloned into pcDNA3.2-DEST using mammalian expression system with Gateway technology (Invitrogen). The nucleic acid sequence of the expression vector was confirmed to be a perfect match at the amino acid level with the FN3K sequence submitted to GenBank™ (accession number NM_022158).

The above expression vectors were transfected into HEK293 cells at 80% confluence using Lipofectamine Plus (Invitrogen) according to the manufacturer's protocol. Transfected cells were cultured for 27 h, washed twice with PBS, and lysed with 80 mM HEPES, pH 7.4, containing CelLytic-M (Sigma), 1 mM DTT, and a protease inhibitor mixture (complete EDTA-free). The cell extracts were centrifuged, and the supernatants (cell lysates) containing FN3K or FN3K-RP were used in additional assays of the phosphorylation of CS-0777 and related compounds.

Statistical Analysis—Comparisons of two groups were analyzed using two-tailed unpaired Student's *t* tests.

RESULTS

CS-0777 Phosphorylation Is Independent of SPHK1 and SPHK2—We first considered whether CS-0777 and structurally related compounds were phosphorylated by SPHK1 or SPHK2, because these enzymes are responsible for phosphorylation of sphingosine to S1P and phosphorylation of the prodrug FTY720 to its active metabolite. We first prepared cells stably expressing human SPHK1 and SPHK2, but the cytosolic fractions obtained from these cells showed only slight phosphorylation activity for CS-0777 (data not shown), suggesting that a different kinase was responsible for the activation/phosphorylation of CS-0777.

We then investigated the source of CS-0777 kinase activity in human blood, because FTY720 kinase activity was reported to be concentrated in platelets (33, 34). Interestingly, CS-0777 kinase activity was found only in the RBC fraction, with little or no kinase activity measurable in platelets, plasma, or lymphocytes (Fig. 2*A*). In addition, the amount of CS-0777 kinase activity in RBCs was comparable with that in whole blood, suggesting the majority of CS-0777 kinase activity in blood was located in the RBC fraction. These findings further indicated a potential difference between CS-0777 and FTY720 kinase activities.

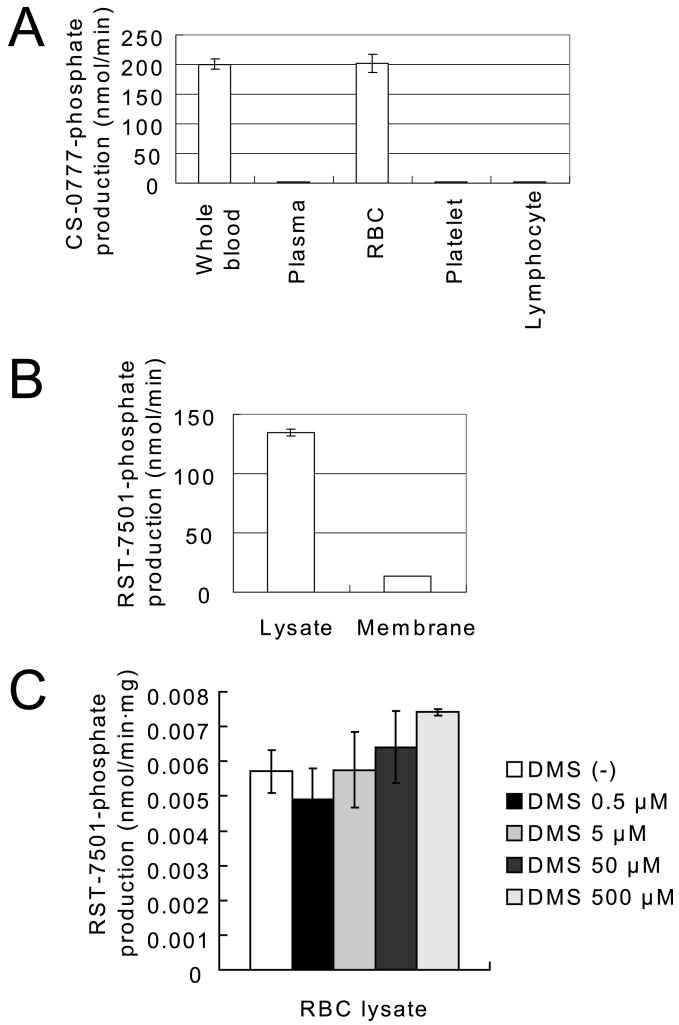

FIGURE 2. **Characterization of CS-0777 and RST-7501 kinase activity in human blood.** *A,* human blood from healthy volunteers was fractionated into plasma, RBC, platelets, and lymphocytes. Whole blood and plasma were diluted 1.1-fold. RBCs, platelets, and lymphocytes were adjusted to a concentration consistent with that in 1.1-fold diluted blood. Production of CS-0777-phosphate was measured following incubation of each sample with CS-0777 (100 μg/ml) for 3 h. *B,* RBCs were lysed and separated into soluble (lysate) and membrane fractions. Membrane precipitate was suspended in buffer containing 20 mM HEPES, pH 7.4, 138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, and 1 mg/ml glucose of the same volume of the lysate. Production of RST-7501-phosphate was measured following incubation of each sample with RST-7501 (10 μg/ml) for 1 h. RST-7501 kinase activity was found mainly in the lysate fraction. *C,* human RBC lysates were tested for RST-7501 kinase activity in the presence or absence of DMS, a specific inhibitor of sphingosine kinases. There was no significant effect of DMS on the production of RST-7501-phosphate. Concentration of RST-7501 was 10 μg/ml, and the reaction time was 3 h. Results in all panels are expressed as means ± S.D. ($n = 3$).

N,N-Dimethylsphingosine (DMS) is known to inhibit both SPHK2 and SPHK1 activity (8, 35). Therefore, we considered whether DMS would inhibit phosphorylation of CS-0777 in human blood. We chose RST-7501, a close structural derivative of CS-0777, for this and subsequent experiments because of its strong UV absorbance, which allows for sensitive and rapid monitoring without requirement for LC-MS. As for CS-0777, RST-7501 kinase activity was mainly observed in the RBC but not in platelets or lymphocytes (data not shown). The RST-7501 kinase activity was predominantly in the soluble lysate fraction compared with the membrane fraction of RBC (Fig. 2*B*). We found that RST-7501 kinase activity in the RBC lysate was not inhibited in the presence of DMS up to 500 μM, a concentration at which both SPHK1 and SPHK2 are inhibited (Fig. 2*C*). Together with the results described above, these data strongly suggested that SPHK1 and SPHK2 were not responsible for the phosphorylation of CS-0777 or structurally related compounds and that a different kinase must be involved.

Purification and Identification of FN3K as a Candidate CS-0777 Kinase—To identify potential kinases responsible for CS-0777 activation, we purified RST-7501 kinase activity from RBC lysates using a stepwise chromatographic approach. Preliminary experiments revealed the following. 1) The activity started to precipitate at 10% saturation with ammonium sulfate, and most of the activity was precipitated at 50% saturation. 2) The activity was inhibited by EDTA. 3) EDTA inhibition was rescued by adding an excessive amount of Mg^{2+}. 4) Iodoacetamide inactivated the activity, which indicated that the reducing conditions were preferable for maintaining activity. 5) The activity was inactivated under acidic conditions (pH ≤5) and was stable under basic conditions (up to pH 10). 6) The estimated molecular weight by gel filtration chromatography was 20–32 kDa. 7) The detergent CHAPS stabilized the activity (data not shown).

Taking advantage of these biochemical properties, we purified the RST-7501 kinase activity by ~10,000-fold using seven successive purification steps (Fig. 3*A*). We chose HIC as the first step because it had the greatest purification yield among the various methods tested in preliminary experiments (data not shown). RST-7501 kinase activity was detected as a single peak following each chromatography step (data not shown). Using SDS-PAGE, we identified a band at 35 kDa that correlated with RST-7501 kinase activity following the sixth (supplemental Fig. 2, *A* and *B*) and the final (Fig. 3, *B* and *C*) purification steps. We analyzed this band by nano-LC-MS/MS, and fructosamine 3-kinase (FN3K, National Center for Biotechnology Information accession number 11545906) was identified with a highly confident overall Mascot score of 164, based on tandem mass spectra (MS/MS) identification of five peptides (supplemental Fig. 2*C*).

Identification of FN3K-RP as the Principal CS-0777 Kinase in Blood—To assess the contribution of FN3K to phosphorylation of CS-0777 and structurally related compounds, we investigated the effect of a competitive inhibitor of FN3K, DMF (36), on RST-7501 kinase activity in RBC lysates and partially purified material from the initial purification steps (Fig. 4*A*). We were surprised to find that DMF did not appreciably inhibit the kinase activity in RBC lysates or the ammonium sulfate precipitate, whereas DMF did inhibit kinase activity in fractions obtained following the initial HIC purification step (Fig. 4*A*). This observation suggested that the enzyme purified using the above scheme was FN3K but that another kinase (or kinases) insensitive to DMF probably existed and principally contributed to the activity, and this other kinase(s) was likely depleted or inactivated by the HIC purification step.

To identify this other kinase(s), we excluded the HIC purification step and found in exploratory studies that anion-exchange chromatography at pH 9.0 was able to separate DMF-sensitive and -insensitive kinase activity. RST-7501 kinase activity bound tightly to the anion-exchange column at pH 9.0

A

Step	Protein conc. [μg/ml]	Activity [U/ml][1]	Volume [ml]	Total protein [mg]	Total activity [U]	Specific activity [U/mg]	Overall fold change	Overall recovery [%]
Lysate	83,000	3.2	50	4,100	160	0.039	1.0	100
1) $(NH_4)_2SO_4$ precipitation	9,000	7.3	50	450	367	0.81	21	230
2) Hydrophobic interaction	170	1.7	60	10	100	10	260	63
3) Blue dye affinity	870	5.1	4.0	3.5	20	5.9	150	13
4) Anion exchange	210	2.1	10	2.1	21	10	260	13
5) Cation exchange	87	2.8	4.0	0.35	11	32	820	6.9
6) Cation exchange	10	7.1	0.20	0.0020	1.4	710	18,000	0.88
7) Gel filtration	4.0	1.6	0.10	0.00040	0.16	410	10,000	0.10

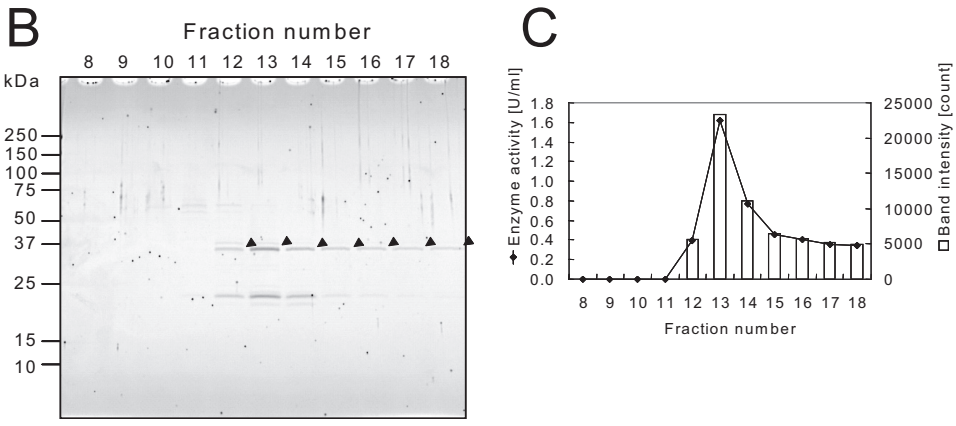

FIGURE 3. **Purification of FN3K from human RBCs.** *A,* steps in the purification of FN3K, starting from human RBC lysate. [1], 1 unit/ml activity was defined as the concentration required for production of 1 μg/ml RST-7501-phosphate (see "Experimental Procedures"). *B,* RST-7501 kinase activity was purified; the active fractions in the final purification step were subjected to SDS-PAGE, and the gel was stained using a fluorescent dye. Bands at 35 kDa (indicated by *arrowheads*) correlated with kinase activity. *C,* kinase activity (*solid line*) and fluorescent intensity of bands shown in *B* (*bars*).

and formed a single broad activity peak on elution. Kinase activity in the initial fractions from this peak was insensitive to DMF, whereas activity in fractions obtained from the tail of the peak showed sensitivity to DMF (Fig. 4*B*).

Thus, to purify the DMF-insensitive kinase, we exchanged HIC for anion-exchange chromatography at pH 9.0 as the first step, and the kinase activity in the eluted fractions was measured with and without DMF. Fractions containing DMF-insensitive activity were collected and subjected to subsequent purification steps. DMF-insensitive RST-7501 kinase activity formed a single peak after each chromatography step (data not shown). As a result of five purification steps, the activity was purified more than 16,000-fold (Fig. 5*A*), and a band at 33 kDa was identified by SDS-PAGE that correlated well with the DMF-insensitive activity following the fourth (supplemental Fig. 3, *A* and *B*) and final purification steps (Fig. 5, *B* and *C*). Nano-LC-MS/MS identified this protein as FN3K-RP (National Center for Biotechnology Information accession number 13959372) with a highly confident overall Mascot score of 209, based on MS/MS identification of seven peptides (supplemental Fig. 3*C*).

Assessment of Kinase Activity for CS-0777 and Related Compounds Using Recombinant FN3K and FN3K-RP—To determine the role of human FN3K and FN3K-RP in the phosphorylation of CS-0777 and related compounds, including FTY720 and sphingosine, we expressed recombinant full-length FN3K and FN3K-RP in a human embryonic kidney cell line (HEK293) and tested cell lysates in our kinase activity assay. Significant RST-7501 kinase activity was observed using lysates from cells overexpressing FN3K and FN3K-RP as compared with lysates from untransfected cells (Fig. 6*A*). Lysates from these cells were also able to induce phosphorylation of FTY720, but the relative activity for FTY720 was extremely weak (Fig. 6*B*, note difference in *y* axis scale with Fig. 6*A*). Neither cell lysate could significantly induce phosphorylation of sphingosine compared with the negative control (Fig. 6*C*).

We assessed the kinase activity of rat RBC lysates and lysates from cells expressing human FN3K and FN3K-RP on 17 CS-0777-related compounds (supplemental Fig. 1), including CS-0777 itself and ROX-2127, another $S1P_1$-selective agonist with a chemical structure similar to CS-0777. Cell lysates from FN3K- and FN3K-RP-transfected cells phosphorylated CS-0777, ROX-2127, and other CS-0777-related compounds, with a similar preference for phosphorylation by FN3K-RP among compounds (supplemental Fig. 5, *A* and *B*). The relative degree of phosphorylation and preference for FN3K-RP in transfected cell lysates was similar to that for rat erythrocytes (supplemental Fig. 5*C*). Correlations between the amount of phosphorylated product obtained using rat RBCs and FN3K- and FN3K-RP-transfected cell lysates were very high (FN3K-RP, $r^2 = 0.8153$, and FN3K, $r^2 = 0.7737$; shown in supplemental Fig. 6), suggesting that FN3K-RP is principally responsible for

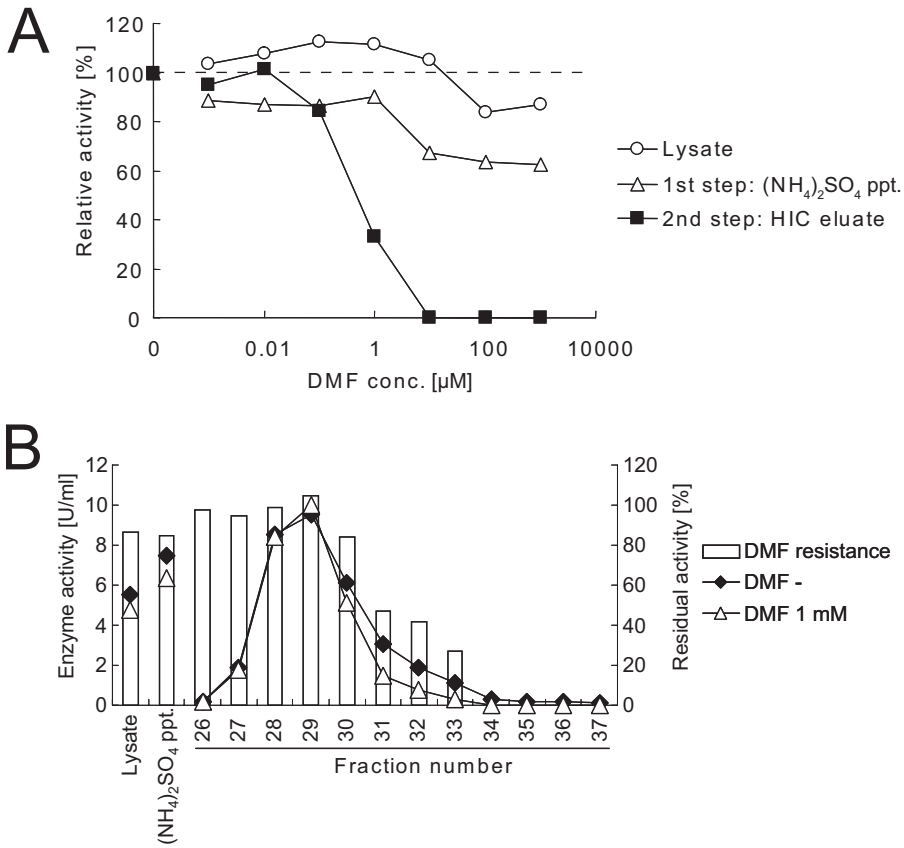

FIGURE 4. **Characterization of DMF-sensitive and -insensitive RST-7501 kinase activity.** *A,* RST-7501 kinase activity in RBC lysate, reconstituted ammonium sulfate precipitate (from the 1st purification step), and active fractions from the second purification step (hydrophobic interaction chromatography), measured in the presence and absence of indicated concentrations (*conc.*) of DMF. Enzyme activity was normalized to that of the sample without DMF. *B,* RST-7501 kinase activity was separated by anion-exchange chromatography at pH 9.0 after ammonium sulfate precipitation of RBC lysate. Activity in the presence and absence of 1 mM DMF is indicated by *open triangles* and *filled diamonds,* respectively. The ratio of the enzyme activity with 1 mM DMF to that without DMF is indicated by *bars* and is expressed as residual activity (%).

phosphorylation of CS-0777 and structurally related compounds in blood. The substrate preferences of FN3K, FN3K-RP, and rat RBC lysates also suggested that introduction of a substituted group on 3-carbon of *N*-methylpyrrole or thiophen diminished phosphorylation efficiency (detailed structure-activity relationships will be described elsewhere).

DISCUSSION

In this study, we identified the activating enzymes of CS-0777, a selective $S1P_1$ modulator prodrug, in human blood. We found that CS-0777 kinase activity was distinct from that for FTY720, which was reported to be phosphorylated predominantly by SPHK2 in platelets (33). We undertook a biochemical strategy coupled with proteomics technology to identify the kinase(s) responsible for CS-0777 phosphorylation in blood. Surprisingly, the kinases purified from human RBCs, FN3K and FN3K-RP, are known to phosphorylate protein-bound sugar moieties that are structurally distinct from the hydrophobic molecules represented by CS-0777 and related compounds (Figs. 3 and 5). Experiments using recombinant FN3K and FN3K-RP suggested FN3K-RP is the principal kinase responsible for phosphorylation of CS-0777 in blood, with significantly less activity toward FTY720 and no activity on sphingosine (Fig. 6). Correlations between substrate preferences using a large number of CS-0777-related compounds also support FN3K-RP as the principal kinase responsible for activating CS-0777 in blood (supplemental Fig. 6).

We purified FN3K and FN3K-RP from small amounts of human RBC lysates by more than 10,000- and 16,000-fold, respectively. The original biochemical purification of FN3K from human erythrocytes was performed by monitoring fructosamine kinase activity, with much larger amounts of starting material. Delpierre *et al.* (37) purified FN3K 2,500-fold, starting with 4,970 ml of human hemolysate, using successive chromatography steps of dye affinity, anion-exchange, and gel filtration. Szwergold *et al.* (38) purified FN3K from 160 g of human RBC lysate by successive chromatography steps of anion exchange, gel filtration, affinity chromatography, and isoelectric focusing. Both FN3K purification schemes were similar to ours, except that we used HIC as the first step, and we started with only 5.2 g of human RBC lysate. Significant improvements in mass spectrometry-based proteomics technology allowed us to identify the candidate protein(s) from SDS-PAGE bands containing as little as nanograms of protein, without requiring purification to homogeneity. Using a similar strategy, our group successfully purified and identified platelet-derived growth factor BB as a new osteoblast differentiation inhibitory factor, starting with conditioned medium from cultured osteoclasts (39), and a key enzyme involved in the interferon-induced anti-

A

Step	Protein conc. [μg/ml]	Activity [U/ml][1]	Volume [ml]	Total protein [mg]	Total activity [U]	Specific activity [U/mg]	Overall fold change	Overall recovery [%]	Residual Activity [%][2]
Lysate	100,000	3.0	50	5,200	150	0.029	1.0	100	81
1) $(NH_4)_2SO_4$ precipitation	9,100	5.0	50	460	250	0.55	19	170	89
2) Anion exchange	640	5.5	10	6.4	55	8.7	300	36	98
3) Blue dye affinity	310	10	1.0	0.31	10	32	1,100	6.6	110
4) Cation exchange	5.0	8.5	0.20	0.00010	1.7	1700	58,000	1.1	98
5) Gel filtration	3.0	1.4	0.10	0.00030	0.14	450	16,000	0.090	n.d.[3]

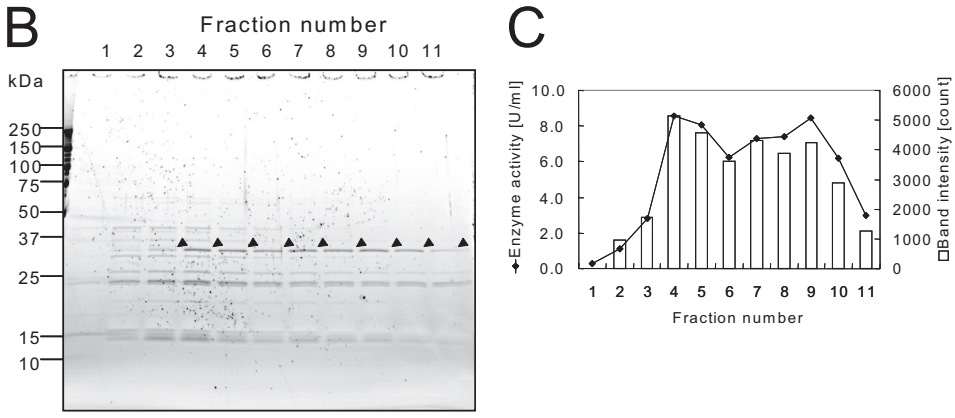

FIGURE 5. **Purification of FN3K-RP from human RBCs.** *A,* steps in the purification of FN3K-RP, starting from human RBC lysate. [1], 1 unit/ml activity was defined as the concentration required for production of 1 μg/ml RST-7501-phosphate. [2], residual activity was defined as the ratio of activity in the presence of 1 mM DMF to that in its absence. [3], *n.d.* means not determined. *B,* active and surrounding fractions from the final purification step were subjected to SDS-PAGE, and the gel was stained using a fluorescent dye. Bands at 33 kDa (*arrowheads*) correlated with kinase activity. *C,* kinase activity (*solid line*) and band intensity (*bars*) are shown for respective fractions.

viral 2–5A system, starting from bovine liver (40). We anticipate that this strategy, using a combination of conventional biochemistry and cutting edge technology, will contribute to discoveries in a wide range of research activities in medicine and biology.

The identification of FN3K and FN3K-RP as CS-0777 kinases was entirely unexpected. FN3K is a 309-amino acid protein that phosphorylates fructosamines, ribulosamines, erythrulosamines, and psicosamines (36, 37, 41, 42). The principal physiological substrate of FN3K is believed to be glycated proteins containing fructosamine, produced via spontaneous interaction between a carboxyl group of glucose and a primary amine on a lysine residue or an N-terminal amino acid (*i.e.* protein glycation). The resulting Schiff base undergoes Amadori rearrangement to produce a protein with covalently bound fructosamine (supplemental Fig. 7). Phosphorylation of the fructosamine residue by FN3K results in an unstable moiety that spontaneously degrades to 3-deoxyglucosone, inorganic phosphate, and the original protein-associated primary amine (41, 42). This has led to the hypothesis that FN3K is a protein-repair enzyme, responsible for "deglycating" proteins and preventing formation of advanced glycation end products, which may play an important role in diabetic complications (41, 42).

FN3K-RP is also a 309-amino acid protein with high homology to FN3K in humans (64% identity at the amino acid level) (43). FN3K-RP phosphorylates ribulosamines, erythrulosamines, psicosamines, and glucitolamines but, unlike FN3K, does not phosphorylate fructosamines (41, 44). FN3K-RP is not inhibited by DMF, a competitive inhibitor of FN3K (43). The physiological role of FN3K-RP is not fully appreciated at present, but similarly to FN3K, it is thought to contribute to repair of glycated proteins containing ribulosamine, erythrulosamine, or psicosamine (formed through protein glycation by ribose 5-phosphate and erythrose 4-phosphate and isomerization from fructosamine, respectively) (41, 42, 45, 46). Despite the relatively low levels of these rare ketoamines relative to fructosamine, the activity of FN3K-RP is severalfold higher than that for FN3K in red blood cells (45), suggesting there may be unknown substrates for FN3K-RP. Neither FN3K nor FN3K-RP has been reported to phosphorylate lipids or drugs, and there are no other reports concerning these enzymes in the context of lipid or drug metabolism. The discovery that FN3K and FN3K-RP are able to phosphorylate small hydrophobic compounds such as CS-0777 may reveal novel roles of these enzymes beyond that of protein deglycation. A search for other endogenous or alimentary substrates for FN3K and FN3K-RP, based on our findings, might lead to discovery of additional physiological roles of these kinases.

Most drugs are considered xenobiotic and undergo metabolism through similar pathways. Although numerous enzymes involved in drug metabolism, such as the cytochrome P450s, have been well studied and are routinely evaluated in the assessment of new drugs, other drug-metabolizing enzymes remain unidentified. In particular, knowledge of the enzymes involved in the metabolic activation of prodrugs is critical for understanding the pharmacokinetic and pharmacodynamic proper-

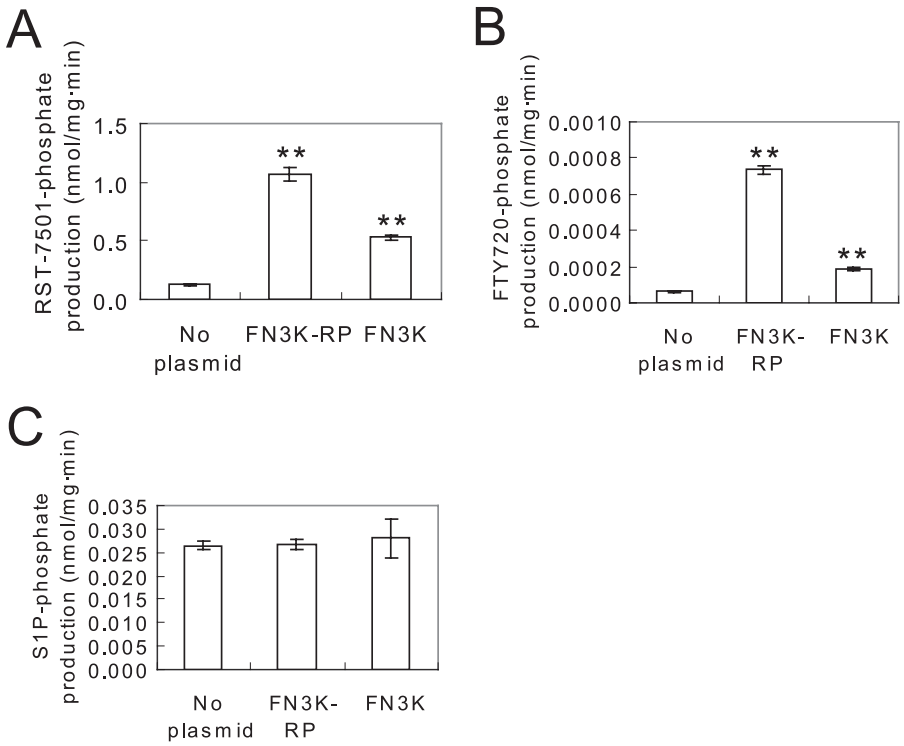

FIGURE 6. **RST-7501, FTY720, and sphingosine kinase activity in lysates from FN3K- and FN3K-RP-transfected cells.** HEK293 cells were transiently transfected with a vector encoding human FN3K-RP or FN3K. The transfectants and HEK293 cells without plasmid (*no plasmid*) were lysed, and lysates were assayed for their ability to phosphorylate RST-7501 (*A*), FTY720 (*B*), and sphingosine (*C*). Results are expressed as means ± S.D. ($n = 3$). *Double asterisks* indicate $p < 0.01$ compared with no plasmid. The concentration of RST-7501 was 100 μg/ml; concentrations of FTY720 and sphingosine were each 10 μM. The reaction time was 3 h. Western blots of those overexpressed cell lysates are shown in supplemental Fig. 4.

ties of such a drug. Because activation pathways may be unique for each prodrug, the enzyme(s) involved may be difficult to anticipate based on the drug's chemical structure or activity. The recent identification of carboxymethylenebutenolidase as an activating enzyme for olmesartan medoxomil (an angiotensin receptor blocker used in treatment of hypertension) is another example of an unexpected prodrug activation pathway (31). We believe it would have been impossible to predict FN3K and FN3K-RP as candidates for CS-0777 activation or phosphorylation using *in silico* approaches. Thus, an unbiased approach, including biochemical purification and expression cloning, may be necessary to identify metabolic enzymes, especially those involved in prodrug activation.

We have demonstrated that the kinases responsible for FTY720 and CS-0777 activation in blood are distinct based on differences in their cellular distribution (Fig. 2*A*) and inhibitor sensitivity (Fig. 2*C*) and ultimately by biochemical purification of FN3K-RP as the principal enzyme involved in CS-0777 phosphorylation in human RBCs. Interestingly, the blood concentration of CS-0777-phosphate in a phase I study in healthy volunteers was more than 20-fold higher than that of CS-0777 (27), whereas blood concentrations of FTY720 and FTY720-phosphate are reported to be roughly equal (47), suggesting differences in the phosphorylation kinetics of the two drugs. Thus, in addition to $S1P_1$ selectivity, a more efficient phosphorylation of CS-0777 could contribute to improved pharmacological or therapeutic properties.

Our data indicate that FN3K-RP is the principal enzyme responsible for CS-0777 activation in human blood, whereas FN3K has a minor role, based on three observations as follows: (i) 80–90% of the activity in RBC lysates was insensitive to DMF, an FN3K-specific inhibitor (Fig. 4); (ii) the DMF-insensitive activity formed a single peak in all the purification steps for FN3K-RP (Fig. 5, supplemental Fig. 3, and data not shown); and (iii) the substrate preference for both FN3K-RP and FN3K showed good correlation with that of rat RBC lysates (supplemental Fig. 5 and Fig. 6). In addition, preliminary experiments indicated that K_m values for CS-0777 kinase activity from RBC lysates and lysates from FN3K-RP- and FN3K-transfected cells were in the same concentration range, supporting FN3K-RP as the principal CS-0777 kinase (data not shown). We investigated whether 1-deoxy-1-morpholinopsicose, reported to be a competitive inhibitor for both FN3K-RP and FN3K (45), could inhibit RST-7501 kinase activity in lysates from FN3K-RP- or FN3K-transfected cells. However, 10 mM 1-deoxy-1-morpholinopsicose did not inhibit RST-7501 kinase activity (data not shown), possibly due to the difference between substrates and the relatively low K_m values for these enzymes (43).

Our focus in this study was to determine the kinase(s) responsible for phosphorylation of CS-0777 in blood, because S1P and fingolimod are both reported to be produced, stored, and released in blood (34). We cannot exclude the possibility that other tissues, including other blood cells, can contribute to activation of CS-0777 *in vivo*. FN3K is expressed in multiple tissues, and FN3K-RP expression was observed in nearly all tissues examined (except for skeletal muscle and intestinal mucosa) (43, 45, 46). A hematopoietic source and a distinct radiation-resistant source have been reported to contribute to

blood and lymph S1P, respectively (48), and the resulting compartmentalization of S1P has an important role in lymphocyte trafficking. Whether similar compartmentalization may exist for phosphorylated metabolites of CS-0777 or FTY720 is currently unknown and deserves further investigation. Studies involving conditional ablation of FN3K-RP in hematopoietic tissues (using radiation) or tissue-specific knock-out of FN3K-RP (using CRE recombinase) in mice would be useful to address these questions.

We have not studied mechanisms for transport of CS-0777 or CS-0777-phosphate across RBC membranes. Despite the importance of S1P as a signaling molecule, the mechanisms of its export from RBCs and other cells are still unclear. Several ABC transporters have been suggested as possible S1P transporters, but S1P concentrations in blood are normal when these transporters are knocked out in mice (49). A novel ATP-dependent S1P transporter that does not require ATP hydrolysis was recently reported in rat erythrocytes (50). This same group also identified spinster-like protein 2 (SPNS2) as a transporter for both S1P and FTY720-phosphate (23, 51), but the expression of SPNS2 is low in hematopoietic and vascular tissues, as well as in megakaryoblast and erythromyeloblastoid leukemia cell lines, suggesting SPNS2 may not be responsible for S1P transport in RBCs. Whether ABC transporters, SPNS2, or some other membrane transporter may be involved in the export of CS-0777-phosphate from RBCs requires further investigation.

Our data implicate RBCs as a likely determinant of blood levels of CS-0777-phosphate *in vivo* and identify FN3K-RP as the principal kinase involved in CS-0777 activation in blood. This discovery was unexpected, because sphingosine and FTY720 are both phosphorylated by sphingosine kinases; this finding may help in understanding potential pharmacokinetic and pharmacodynamic differences between CS-0777 and FTY720. Identification of a novel xenobiotic metabolic pathway involving FN3K and FN3K-RP may lead to a better understanding of the biochemical and physiological roles of these enigmatic "housekeeping" enzymes. As FN3K and FN3K-RP also phosphorylated ROX-2127, an S1P receptor modulator with a different chemical scaffold than CS-0777 (supplemental Figs. 1 and 5), these enzymes may contribute to metabolism or activation of other drugs with a potential role in treatment of autoimmune diseases.

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REFERENCES

1. Compston, A., and Coles, A. (2008) *Lancet* **372,** 1502–1517
2. Galetta, S. L., and Markowitz, C. (2005) *CNS Drugs* **19,** 239–252
3. Ransohoff, R. M. (2007) *N. Engl. J. Med.* **356,** 2622–2629
4. Marsolais, D., and Rosen, H. (2009) *Nat. Rev. Drug Discov.* **8,** 297–307
5. Horga, A., Castilló, J., and Montalban, X. (2010) *Expert Opin. Pharmacother.* **11,** 1183–1196
6. Olivera, A., Kohama, T., Tu, Z., Milstien, S., and Spiegel, S. (1998) *J. Biol. Chem.* **273,** 12576–12583
7. Kohama, T., Olivera, A., Edsall, L., Nagiec, M. M., Dickson, R., and Spiegel, S. (1998) *J. Biol. Chem.* **273,** 23722–23728
8. Liu, H., Sugiura, M., Nava, V. E., Edsall, L. C., Kono, K., Poulton, S., Milstien, S., Kohama, T., and Spiegel, S. (2000) *J. Biol. Chem.* **275,** 19513–19520
9. Nava, V. E., Lacana, E., Poulton, S., Liu, H., Sugiura, M., Kono, K., Milstien, S., Kohama, T., and Spiegel, S. (2000) *FEBS Lett.* **473,** 81–84
10. Matloubian, M., Lo, C. G., Cinamon, G., Lesneski, M. J., Xu, Y., Brinkmann, V., Allende, M. L., Proia, R. L., and Cyster, J. G. (2004) *Nature* **427,** 355–360
11. Allende, M. L., Dreier, J. L., Mandala, S., and Proia, R. L. (2004) *J. Biol. Chem.* **279,** 15396–15401
12. Schwab, S. R., Pereira, J. P., Matloubian, M., Xu, Y., Huang, Y., and Cyster, J. G. (2005) *Science* **309,** 1735–1739
13. Brinkmann, V., Billich, A., Baumruker, T., Heining, P., Schmouder, R., Francis, G., Aradhye, S., and Burtin, P. (2010) *Nat. Rev. Drug Discov.* **9,** 883–897
14. Fujita, T., Hirose, R., Yoneta, M., Sasaki, S., Inoue, K., Kiuchi, M., Hirase, S., Chiba, K., Sakamoto, H., and Arita, M. (1996) *J. Med. Chem.* **39,** 4451–4459
15. Brinkmann, V., Pinschewer, D. D., Feng, L., and Chen, S. (2001) *Transplantation* **72,** 764–769
16. Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G. J., Card, D., Keohane, C., Rosenbach, M., Hale, J., Lynch, C. L., Rupprecht, K., Parsons, W., and Rosen, H. (2002) *Science* **296,** 346–349
17. Brinkmann, V., Davis, M. D., Heise, C. E., Albert, R., Cottens, S., Hof, R., Bruns, C., Prieschl, E., Baumruker, T., Hiestand, P., Foster, C. A., Zollinger, M., and Lynch, K. R. (2002) *J. Biol. Chem.* **277,** 21453–21457
18. Gräler, M. H., and Goetzl, E. J. (2004) *FASEB J.* **18,** 551–553
19. Budde, K. L., Schmouder, R., Nashan, B., Brunkhorst, R. W., Lücker, P., Mayer, T., Brookman, L., Nedelman, J., Skerjanec, A., Böhler, T., and Neumayer, H. H. (2003) *Am. J. Transplant.* **3,** 846–854
20. Böhler, T., Waiser, J., Schuetz, M., Neumayer, H. H., and Budde, K. (2004) *Nephrol. Dial. Transplant.* **19,** 702–713
21. Cohen, J. A., Barkhof, F., Comi, G., Hartung, H. P., Khatri, B. O., Montalban, X., Pelletier, J., Capra, R., Gallo, P., Izquierdo, G., Tiel-Wilck, K., de Vera, A., Jin, J., Stites, T., Wu, S., Aradhye, S., and Kappos, L. (2010) *N. Engl. J. Med.* **362,** 402–415
22. Kappos, L., Radue, E. W., O'Connor, P., Polman, C., Hohlfeld, R., Calabresi, P., Selmaj, K., Agoropoulou, C., Leyk, M., Zhang-Auberson, L., and Burtin, P. (2010) *N. Engl. J. Med.* **362,** 387–401
23. Hisano, Y., Kobayashi, N., Kawahara, A., Yamaguchi, A., and Nishi, T. (2011) *J. Biol. Chem.* **286,** 1758–1766
24. Nishi, T., Miyazaki, S., Takemoto, T., Suzuki, K., Iio, Y., Nakajima, K., Ohnuki, T., Kawase, Y., Nara, F., Inaba, S., Izumi, T., Yuita, H., Oshima, K., Doi, H., Inoue, R., Tomisato, W., Kagari, T., and Shimozato, T. (2011) *ACS Med. Chem. Lett.* **2,** 368–372
25. Sanna, M. G., Liao, J., Jo, E., Alfonso, C., Ahn, M. Y., Peterson, M. S., Webb, B., Lefebvre, S., Chun, J., Gray, N., and Rosen, H. (2004) *J. Biol. Chem.* **279,** 13839–13848
26. Kubota, K., Anjum, R., Yu, Y., Kunz, R. C., Andersen, J. N., Kraus, M., Keilhack, H., Nagashima, K., Krauss, S., Paweletz, C., Hendrickson, R. C., Feldman, A. S., Wu, C. L., Rush, J., Villén, J., and Gygi, S. P. (2009) *Nat. Biotechnol.* **27,** 933–940
27. Moberly, J., Rohatagi, S., Zahir, H., Hsu, C., Noveck, R., and Truitt, K. (2011) *J. Clin. Pharmacol.*, in press
28. Billich, A., Bornancin, F., Dévay, P., Mechtcheriakova, D., Urtz, N., and Baumruker, T. (2003) *J. Biol. Chem.* **278,** 47408–47415
29. Paugh, S. W., Payne, S. G., Barbour, S. E., Milstien, S., and Spiegel, S. (2003) *FEBS Lett.* **554,** 189–193
30. Kubota, K., Wakabayashi, K., and Matsuoka, T. (2003) *Proteomics* **3,** 616–626
31. Ishizuka, T., Fujimori, I., Kato, M., Noji-Sakikawa, C., Saito, M., Yoshigae, Y., Kubota, K., Kurihara, A., Izumi, T., Ikeda, T., and Okazaki, O. (2010) *J. Biol. Chem.* **285,** 11892–11902
32. Natsume, T., Yamauchi, Y., Nakayama, H., Shinkawa, T., Yanagida, M., Takahashi, N., and Isobe, T. (2002) *Anal. Chem.* **74,** 4725–4733
33. Anada, Y., Igarashi, Y., and Kihara, A. (2007) *Eur. J. Pharmacol.* **568,**

106–111
34. Kihara, A., and Igarashi, Y. (2008) *Biochim. Biophys. Acta* **1781,** 496–502
35. Edsall, L. C., Van Brocklyn, J. R., Cuvillier, O., Kleuser, B., and Spiegel, S. (1998) *Biochemistry* **37,** 12892–12898
36. Delpierre, G., Vanstapel, F., Stroobant, V., and Van Schaftingen, E. (2000) *Biochem. J.* **352,** 835–839
37. Delpierre, G., Rider, M. H., Collard, F., Stroobant, V., Vanstapel, F., Santos, H., and Van Schaftingen, E. (2000) *Diabetes* **49,** 1627–1634
38. Szwergold, B. S., Howell, S., and Beisswenger, P. J. (2001) *Diabetes* **50,** 2139–2147
39. Kubota, K., Sakikawa, C., Katsumata, M., Nakamura, T., and Wakabayashi, K. (2002) *J. Bone Miner. Res.* **17,** 257–265
40. Kubota, K., Nakahara, K., Ohtsuka, T., Yoshida, S., Kawaguchi, J., Fujita, Y., Ozeki, Y., Hara, A., Yoshimura, C., Furukawa, H., Haruyama, H., Ichikawa, K., Yamashita, M., Matsuoka, T., and Iijima, Y. (2004) *J. Biol. Chem.* **279,** 37832–37841
41. Van Schaftingen, E., Delpierre, G., Collard, F., Fortpied, J., Gemayel, R., Wiame, E., and Veiga-da-Cunha, M. (2007) *Adv. Enzyme Regul.* **47,** 261–269
42. Van Schaftingen, E., Collard, F., Wiame, E., and Veiga-da-Cunha, M. (2010) *Amino Acids,* in press
43. Collard, F., Delpierre, G., Stroobant, V., Matthijs, G., and Van Schaftingen, E. (2003) *Diabetes* **52,** 2888–2895
44. Szwergold, B., Manevich, Y., Payne, L., and Loomes, K. (2007) *Biochem. Biophys. Res. Commun.* **361,** 870–875
45. Collard, F., Wiame, E., Bergans, N., Fortpied, J., Vertommen, D., Vanstapel, F., Delpierre, G., and Van Schaftingen, E. (2004) *Biochem. J.* **382,** 137–143
46. Delplanque, J., Delpierre, G., Opperdoes, F. R., and Van Schaftingen, E. (2004) *J. Biol. Chem.* **279,** 46606–46613
47. Kovarik, J. M., Dole, K., Riviere, G. J., Pommier, F., Maton, S., Jin, Y., Lasseter, K. C., and Schmouder, R. L. (2009) *J. Clin. Pharmacol.* **49,** 212–218
48. Pappu, R., Schwab, S. R., Cornelissen, I., Pereira, J. P., Regard, J. B., Xu, Y., Camerer, E., Zheng, Y. W., Huang, Y., Cyster, J. G., and Coughlin, S. R. (2007) *Science* **316,** 295–298
49. Lee, Y. M., Venkataraman, K., Hwang, S. I., Han, D. K., and Hla, T. (2007) *Prostaglandins Other Lipid Mediat.* **84,** 154–162
50. Kobayashi, N., Kobayashi, N., Yamaguchi, A., and Nishi, T. (2009) *J. Biol. Chem.* **284,** 21192–21200
51. Kawahara, A., Nishi, T., Hisano, Y., Fukui, H., Yamaguchi, A., and Mochizuki, N. (2009) *Science* **323,** 524–527