A Silent Mutation Made Possible Efficient Production of Active Human Frk Tyrosine Kinase in *Escherichia coli*

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Fyn-related kinase (Frk) was first identified using human breast cancer cells. It shares 51% identity with c-Src. Like all members of the Src family, Frk is thought to cause several cancers via dysregulations in signal transduction from cell-surface receptors. The excess activity of Frk on β-cells has a crucial role in type-1 diabetes. A silent mutation at Ile229 conferred a bacterial expression system on the kinase domains of Frk, which allowed for the quick expression and purification of one unphosphorylated and two mono-phosphorylated kinase domains. The C-terminal catalytic segment of the human Frk kinase conjugating hexahistidine purification tag (His-tag) was expressed in *Escherichia coli*. After first-step purification utilizing the His-tag, an anion-exchange chromatogram yielded three major peaks that had distinguishable phosphorylation characteristics as judged by Western blot analysis and measurement of kinase activity. This result of active protein production should promote drug discovery studies, including high-throughput screening and structure-based drug design.

Key words: silent mutant; Fyn-related kinase (Frk); bacterial expression

Fyn-related kinase (Frk) was discovered using human breast cancer cells.1,2 DNA sequencing revealed that Frk is a member of the Src family of non-receptor protein kinases.3,4 Like all members of the Src family (comprising Src, Fyn, Yes, Lyn, Lck, Hck, Fgr, and Blk), Frk has functional domain structures containing a unique N-terminus sequence, an SH3 domain, an SH2 domain, a kinase catalytic domain (SH1), and a C-terminus regulatory sequence (Fig. 1). It does not have the N-terminal glycine essential for myristoylation and membrane localization, whereas the other Src family kinases associate with the cell membrane by the myristylated glycine residue.5,6 The fact that Frk does not have the N-terminal glycine and has a putative nuclear localization signal sequence in the SH2 domain is consistent with localization of Frk to the nucleus.7 The SH2 and SH3 domains are important in the recognition of substrate and adaptor proteins, and in the fixation of inactive conformation.8,9 The kinase domain is essential to the enzyme activity of Frk. Phosphorylation at two tyrosine residues corresponding to Tyr387 and Tyr497 of Frk modulates kinase activity in Frk as well as in other Src family kinases.10 Autophosphorylation of Tyr387 in the activation loop of the kinase domain brings about conformational rearrangement of the activation loop, and Frk kinase finally achieves a hyper-activated state.11 Phosphorylation of Tyr497 in the C-terminal sequence by CSK kinase reduces the enzyme activity of Frk due to C-terminal sequence binding to the SH2 domain.12,13

Frk is predominantly expressed in epithelial cells in the kidney, pancreas, intestine, and liver, but not in the lung, muscle, brain, or heart.5,14 Dysregulation of Frk kinase activity causes several diseases. It has been found to be expressed at high levels in a subset of primary human epithelial tumors, including the BT-20 breast cancer cell line and the LS180 colon cancer cell line.15 Constitutively active Frk fused with ETV6 (a protein frequently involved in rearrangements leading to acute myelogenous leukemia) has been identified in patients with chronic myelogenous leukemia.15 It was recently reported that dysregulation of Frk kinase activity caused β cell-destructive processes in type-1 diabetes.16

Obtaining the crystal structure of the human Frk kinase domain might promote detailed understanding of the mechanisms of molecular recognition for the design of high-affinity, selective inhibitors. The ability to produce highly purified kinases in amounts sufficient for crystallography is currently limited. Kinases exist in various conformational states corresponding to phosphorylation states. The crystal structures in both states can be used in structure-based drug design, because some kinase inhibitors such as Imatinib and PD173955 prefer to bind to one of the two distinct states of the same kinase. Imatinib and PD173955 bind to the intrinsic-active state unphosphorylated and to the hyper-activated state phosphorylated, respectively, of c-Abl kinase.8

Here, we report the expression, purification, and characterization of recombinant human Frk tyrosine kinase obtained by a bacterial expression system.

Materials and Methods

Cloning of the human Frk kinase domain. The C-terminal segment (hFrkC, residues 228–505) comprising the kinase domain and the C-terminal sequence among the full length of human Frk tyrosine kinase was inserted into bacterial expression vector pET30a (Novagen, Darmstadt, Germany) incorporating a C-terminal hexahistidine purification tag (His-tag). The insert sequence for the hFrkA segment, with a
calculated molecular weight of 33,006, was amplified from full-length Frk (NM_002031) inserted into vector pFastBac HTb (Invitrogen, Carlsbad, CA) by the polymerase chain reaction (PCR) method using Ex Taq DNA polymerase (Takara, Kyoto, Japan). The forward primer was 5'-TTCAATATGGAGATACGGCACA-3' (double-underlined nucleotides indicate the location of an Nde I site) and the reverse primer was 5'-TGAATTCCTCACATGTCGTTTGTGGTTAACCTCT-3' (double-underlined nucleotides indicate the location of an EcoR I site, and single-underlined nucleotides encode His-tag). PCR products including hFrkα and His-tag were restricted with Nde I (Takara) and EcoR I (Toyobo, Osaka, Japan). They were ligated into the respective restricted sites of pET30a (Novagen) with a Ligation-Convenience Kit (Nippon Gene, Tokyo, Japan) including T4 DNA ligase overnight at 16 °C. DH5α competent cells (Toyobo) were transformed with the pET30a-Frkα construct. Positive clones were selected in agar medium containing kanamycin (Sigma, St. Louis, MO). The plasmid was purified and insertion of the pET30a-Frkα construct was confirmed by DNA sequencing with a multi-capillary DNA analyzing system CEQ2000 (Beckman Coulter, Fullerton, CA).

Silent mutation at Ile229. Silent-mutated plasmids with rare codon ATA in Escherichia coli, corresponding to Ile229, to preferred codon ATT or ATC in E. coli were prepared by PCR using Pfu turbo polymerase (Stratagene, La Jolla, CA) with primers for the ATT-mutant, 5'-CATATGGAGATACGGCACA-3' (underlined nucleotides indicate the location of Ile229) and for the ATC mutant, 5'-CATATGGAATTACGCGAAC-3' (underlined nucleotides indicate the location of Ile229). To enhance selection of the mutated plasmid from the parental plasmid, the PCR amplification reaction pool was digested with Dpn I (New England BioLabs, Beverly, MA) before transformation into competent cells. The mutated plasmid was amplified in a manner similar to preparation of the intact plasmid. The plasmid was purified, and insertion of the pET30a-Frkα construct confirmed by DNA sequencing, as detailed above.

Expression of the Frk kinase domain in E. coli. Competent cells BL21DE3 (Novagen), BL21DE3-Codon-Plus RP (Novagen), TunerDE3 (Novagen), and Rosetta-gami2DE3 (Novagen) were transformed with intact and mutated plasmids. The transformed cells were plated on LB agar with 50 μg/ml kanamycin (Sigma) and grown overnight at 37 °C. The next day, colonies from the plates were resuspended in LB media with 50 μg/ml kanamycin. Cultures were grown to an OD600 of 0.8 at 37 °C. They were cooled for 1 h at 25 °C before induction for 6 h at 25 °C with 0.5 mM IPTG.

Expression check using spin columns. Expression of hFrkα was checked using His SpinTrap (a spin column containing Ni-sepharose gel for histidine-tagged proteins) according to the manufacturer’s instruction (GE Healthcare, Buckinghamshire, England). Cell pellets were suspended with a binding buffer consisting of 20 mM sodium phosphate, 500 mM NaCl, and 20 mM imidazole at pH 7.4. The cell pellets were lysed by 3 cycles of ultrasonic treatment at 4 °C. The supernatant after centrifugation was applied to the spin column and incubated for 10 min. After washing out the non-binding components, the binding buffer, the expressed Frk protein was eluted with an elution buffer consisting of 20 mM sodium phosphate, 500 mM NaCl and 500 mM imidazole at pH 7.4. Protein was detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

SDS–PAGE and Western blot analysis. Purified hFrkα protein samples were analyzed by SDS–PAGE using 12.5% polyacrylamide gels. The gels were stained with Bio Safe Coomassie (Bio-Rad, Fremont, CA). For Western blots, protein samples were resolved on SDS–PAGE using 12.5% polyacrylamide gel and transferred onto PVDF membrane Hybond-P (GE Healthcare) with an apparatus (Biocraft, Tokyo, Japan) according to the manufacturer’s instructions. The membrane was blocked with 5% skim milk in TBS buffer (20 mM Tris–HCl, pH 7.5, and 140 mM NaCl) for 1 h. The membrane was washed 3 times with TBST buffer (20 mM Tris–HCl (pH 7.5), 140 mM NaCl, and 0.05% Tween-20) and incubated for 1 h with PY20 (Abcam, Cambridge, England) as the anti-phosphotyrosine antibody conjugated alkaline phosphatase with 1:500 dilution. The membrane was washed 3 times with TBST buffer, and bands were visualized by applying a chromogenic alkaline phosphatase substrate using nitroblue tetrazolium blue chloride and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).
Frk kinase activities and kinetic parameters. Frk kinase activities were measured by a method reported for Lyn kinase of Src family kinase. Enzyme reactions were conducted in a 80-μl volume containing hFrk A solution (0–1 ng/μl), 1 μM FITC-labeled substrate peptide, and 800 μM ATP in a reaction buffer (20 mM HEPEs, pH 7.5, 0.01% Triton X-100, 5 mM MgCl₂, and 2 mM DTT) at room temperature. The amounts of unphosphorylated and phosphorylated substrate peptides were repeatedly measured at 5-min intervals up to 70 min by Mobility Shift Micro Fluidic Technology (Caliper LC3000 System, Caliper Life Sciences, Hopkinton, MA). The specific activities of the hFrk A protein were defined by the amount (mole) of phosphate transferred to the FITC-labeled substrate peptides per min per mg of protein.

To determine the Kₘ values of the hFrk A protein for ATP, kinase proteins (1 ng/ml of fraction 1 or 2 and 0.05 ng/ml of fraction 2) and 1 μM FITC-labeled substrate peptide were mixed with ATP (6.25–800 μM) in the reaction buffer at room temperature. Steady-state kinetic parameters were determined by fitting to non-linear least squares analysis of the initial velocity data. The inhibitory effects of staurosporine (Wako, Osaka, Japan) against the hFrk A proteins were determined at the corresponding Kₘ values for ATP.

Autophosphorylation. The autophosphorylation reaction was initiated by adding an equal volume of purified hFrk A protein to a mixture of ATP and MgCl₂, and the reaction was allowed to proceed for 30 min at 30 °C. The reaction concentration for the enzyme, ATP, and MgCl₂ were 0.25 mg/ml, 2.5 mM, and 5 mM respectively. The reaction was detected using Western blot analysis, as detailed above.

Results and Discussion

Expression and purification of protein

The expression construct for hFrk A (Fig. 1) was designed by reference to the crystallizable Fyn and Lyn kinase domains, due to their high primary amino acid sequence homology. SDS–PAGE from the coarse purification by the spin column for His-Tag revealed that the hFrk A protein with the intact DNA sequence of ATA corresponding to Ile229 was barely expressed using any E. coli competent cell, including BL21DE3, BL21DE3-Codon-Plus RP, TunerDE3, and Rosetta-gami 2 DE3. The ATA sequence is a minor isoleucine codon on the gene expression of E. coli, and the minor codons near the start codon negatively affect gene expression. Two silent mutants alternatively used with the preferred codon for isoleucine in the E. coli, the ATT and the ATC sequences were prepared for E. coli expression. Expression of the ATT mutant using the competent cell TunerDE3 was detected by SDS–PAGE after the His-tag spin column experiment, even though the expression level of protein was low. The ATT mutant was not expressed using any competent cells. Subsequent mass production of the hFrk A protein was done using the ATT mutant at Ile229 without a further mutation experiment.

The hFrk A protein was purified by the same method as for Fyn or Lyn. Recombinant hFrk A was initially bound onto the Ni-NTA column and eluted at 250 mM imidazole. SDS–PAGE analysis of Ni-purified hFrk A revealed the expected mass corresponding to the recombinant hFrk A construct. Crude hFrk A protein of 3.4 mg with an approximate purity of 60% (as judged with a densitometer) was obtained from 10.9 g of cells. It had kinase activity (Table 1). For the next purification, an anion-exchange column was chosen because the crude sample was likely to contain several phosphorylated states of the hFrk A protein in reference to the findings on Lyn production. Lyn had four characteristic phosphorylated states during expression and purification. Running a shallow 60 column volume NaCl gradient, three independent peaks were resolved by anion-exchange chromatography on a Mono Q column from the Ni-purified hFrk A sample (Fig. 2). SDS–PAGE analysis revealed that these peaks were to be assigned as homologous hFrk A (Fig. 3a). Three hFrk A protein samples with distinct net charges on the protein surface were fractionated. These summed up to an activity yield of 27% by anion-exchange chromatography.

<table>
<thead>
<tr>
<th>Total proteina</th>
<th>Total activityb</th>
<th>Specific activityc</th>
<th>Kₘd</th>
<th>kcat,e</th>
<th>IC₅₀f</th>
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<tr>
<td>Ni-NTA pool</td>
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<td>41,666</td>
<td>12,183</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mono-Q peak 1</td>
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<td>5,422</td>
<td>28,537</td>
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<td>475</td>
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<tr>
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<td>114,337</td>
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<td>1,905</td>
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<tr>
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<td>0.06</td>
<td>1,274</td>
<td>21,231</td>
<td>118</td>
<td>353</td>
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</table>

As well as anion-exchange chromatography, Western blotting experiments using the anti-phosphotyrosine antibody suggested that the three fractionated samples probably represented different phosphorylation states of purified recombinant hFrk A. The unphosphorylated sample (Fig. 3a) was eluted as the first peak on anion-exchange chromatography. Phosphorylated samples as peaks 2 and 3 (Fig. 3a) were subsequently eluted (Fig. 2). Using a steeper gradient, such as a 30 column volume NaCl gradient, peaks 2 and 3 could not be resolved, although peak 1 could. This result suggests that the two peaks probably had similar negative net charges on the protein surface.

The three fractioned hFrk A had kinase activities for a substrate peptide, and were suppressed by the broad-spectrum kinase inhibitor staurosporine (Table 1). Al-
though these fractioned hFrk\(^\Delta\) revealed approximately 10-fold lower activity than the Lyn samples prepared in the previous study,\(^9\) the result suggested that the fractioned samples had intact activities: enzyme activity of the hFrk\(^\Delta\) sample as peak 2 was comparable to the Frk sample prepared using insect cells (Carna Biosciences, Kobe, Japan).

Unphosphorylated hFrk\(^\Delta\) (peak 1) showed intrinsic enzyme activities in the phosphorylation of the substrate peptide (Table 1) and in autophosphorylation (Fig. 3b). Identical activities have been observed in living cells.\(^13\)

The phosphorylated protein as peak 2 revealed maximal activity for the substrate peptide (Table 1). This sample was probably in a hyper-activated state, which is mono-phosphorylated at Tyr387 in the activation loop. It would appear that the other phosphorylated protein as peak 3 was mono-phosphorylated at Tyr497, because no other phosphorylation site in the expression construct has been reported and the phosphorylation had little effect on enzyme activity. The enzyme activity of the phosphorylated protein as peak 3 was similar to that of the unphosphorylated protein (Table 1). The phosphorylation at Tyr497 in the truncated expression construct had no significant effect on enzyme activity because the phospho-Tyr497 residue interacts with the SH2 domain of the full-length Frk to be suppressed as the inactive state. Biochemical analyses of the purified samples reveals that the intrinsic-active and hyper-activated states of hFrk\(^\Delta\) were prepared for the crystallization experiments.

In summary, we purified intrinsic-active and hyper-activated hFrk\(^\Delta\) protein samples in sufficient quantities and at sufficient purities for biochemical and crystallographic studies by the bacterial expression system. The ability to generate this protein kinase should be beneficial in future structure–function studies and structure-based drug design initiatives involving this biochemically important kinase.

References