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Expression, purification and preliminary crystallographic studies on the catalytic region of the nonreceptor tyrosine kinase Fes

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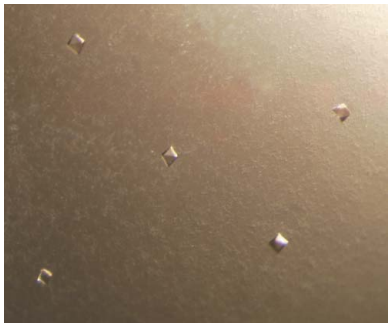

The proto-oncogene tyrosine protein kinase c-*fps*/*fes* encodes a structurally unique protein (Fes) of the nonreceptor protein-tyrosine kinase (PTK) family. Its expression has been demonstrated in myeloid haematopoietic cells, vascular endothelial cells and in neurons. In human-derived and murine-derived cell lines, the activated form of this kinase can induce cellular transformation; moreover, it has been shown that Fes is involved in the regulation of cell–cell and cell–matrix interactions mediated by adherens junctions and focal adhesions. The N-terminus of Fes contains the FCH (Fps/Fes/Fer/CIP4 homology) domain, which is unique to the Fes/Fer kinase family. It is followed by three coiled-coil domains and an SH2 (Src-homology 2) domain. The catalytic region (Fes-CR) is located at the C-terminus of the protein. The successful expression, purification and crystallization of the catalytic part of Fes (Fes-CR) are described.

1. Introduction

The nonreceptor tyrosine protein kinase Fes (Fes; EC 2.7.10.2) is a 92 kDa protein encoded by the proto-oncogene c-*fps*/*fes* and together with the Fps (Fujinami poultry sarcoma)/Fes-related protein Fer it belongs to a unique subclass of the protein tyrosine kinase (PTK) family (Roebroek *et al.*, 1985; Wilks & Kurban, 1988; Hao *et al.*, 1989). These two proteins are characterized by the presence of an FCH (Fes/Fer/CIP4 homology) domain that is involved in binding to microtubules (Aspenström, 1997). Importantly, the presence of such a domain is consistent with the possible involvement of Fes and Fer in cytoskeletal rearrangement, receptor inside-out signalling and vesicular transport (Tian *et al.*, 2000). Another important feature of Fes/Fer family kinases is the lack of both the SH3 domain and the negative regulatory tyrosine-phosphorylation site in the carboxyl-terminal region. This, together with the presence of an FCH region, distinguishes Fes and Fer from other nonreceptor PTKs such as the members of the Src kinases family.

Fes was first identified as the product of the retroviral v-*fps* oncogene responsible for avian and feline tumours (Snyder & Theilen, 1969; Shibuya *et al.*, 1980). This viral oncogene encodes a chimeric protein, Gag-Fes, consisting of the N-terminal viral protein Gag sequence fused to Fes. Gag-Fes has an unregulated tyrosine kinase activity that abrogates the need for cytokines in the differentiation of hematopoietic progenitor cells (Carmier & Samarut, 1986; Meckling-Gill *et al.*, 1992). In humans, the expression of Fes was initially detected in myeloid haematopoietic cells (MacDonald *et al.*, 1985; Feldman *et al.*, 1985), where it is believed to participate in the regulation of survival and terminal differentiation. Moreover, Fes is also expressed in vascular endothelial, epithelial and neuronal cells, where it plays an important role in angiogenesis (Greer *et al.*, 1994; Haigh *et al.*, 1996).

Several proteins have been identified as putative substrates for both Gag-Fes and human Fes kinase. These are the p120RasGAP protein (Koch *et al.*, 1989; Ellis *et al.*, 1990; Moran *et al.*, 1991), phosphatidylinositol 3-kinase (Fukui *et al.*, 1991), the break-point cluster region (BCR) protein (Maru *et al.*, 1995), STAT3 (Garcia *et al.*, 1997) and connexin43 (Kurata & Lau, 1994). However, the cellular transformation by Fes is not yet fully understood.

The suggested involvement of Fes in cytoskeleton remodelling led our group to a better characterization of its expression and function in model systems other than blood cells, such as neurons, in which motility and interactions with the extracellular matrix are crucial for several biological processes. Intriguingly, while Fes is not detected in early-stage neural stem cells (NSCs), it is significantly expressed by committed neurons (but not glial cells) in several areas such as the hippocampus, cerebellum, striatum and dorsal root ganglia (Condorelli, 2006). This may imply that the acquired expression of Fes has a role in determining specific neuronal commitments. High levels of Fes expression are also detected in four different neuroblastoma cell lines (SK-N-BE, SH-SY5Y, LAN5 and IMR-32). In these cell lines, we found a constitutive expression and activation of Fes, weakly modulated by cell adhesion to various substrates (fibronectin, laminin, b1- or a/v-specific antibodies). Moreover, all these neuroblastoma cell lines displayed a marked resistance to anoikis (80% viability after 48 h in poly-HEMA-coated dish), a typical feature of neoplastic cells (Picco *et al.*, submitted).

Fes consists of a single polypeptide chain folded into five domains: an N-terminal FCH (Fps/Fes/Fer/CIP4 homology) domain, which is unique to the Fes/Fer kinase family, three coiled-coil domains and an SH2 (Src-homology 2) domain. The catalytic domain (Fes-CR) is located at the C-terminus of the protein. The NMR structure of the SH2 domain is already available (PDB code 1wqu; Scott *et al.*, 2005). In this manuscript, we describe the subcloning, expression, purification and crystallization of the catalytic domain of Fes, Fes-CR. Because of its direct catalytical role, this domain is the best target for designing specific inhibitors with potential therapeutic applications. Fes-CR consists of 262 amino acids, corresponding to residues 561–822 of Fes, and has an expected molecular weight of 29 502.88 Da, as calculated using the protein-analyis tools of the *ExPASy* server (Gasteiger *et al.*, 2003).

2. Cloning, expression and purification of the Fes tyrosine kinase domain in *E. coli*

The DNA sequence of the human c-*fps*/*fes* gene (NM_002005, NCBI) coding for the Fes-CR domain was amplified by PCR using the pEYFP-WT-Fps/Fes plasmid as a template (a kind gift from Dr T. Smithgall, University of Pittsburgh, Pittsburgh, USA). In this process, a forward primer for the nucleotides between positions 1741 and 1762 on the c-*fps*/*fes* cDNA was paired with a reverse primer complementary to the nucleotides between positions 2520 and 2541 at the 3′-end of the gene (Sigma Genosys), thus allowing specific amplification of the catalytic region (Fes-CR) of the protein. A FLAG-tag sequence was fused in frame to the 3′ end of the gene in order to facilitate protein purification. *Kpn*I and *Hin*dIII sites were also included in the oligonucleotide chains of the forward and reverse primers, respectively, to allow the cloning procedure. By digesting both the amplified DNA and the pQE30 expression vector (Qiagen) carrying the gene for ampicillin resistance with the *Kpn*I and *Hin*dIII restriction enzymes, the Fes-CR-FLAG sequence was subcloned in frame with the six-histidine (6×His) cassette of this plasmid located at the 5′-end of the insert (the N-terminus of the expressed protein). DH-5α *E. coli* competent cells (Invitrogen) were transformed with the constructs and the resulting clones were screened by PCR. Those incorporating the 6×His-Fes-CR-FLAG insert were then sequenced (MWG) in order to exclude the possibility of point mutations or frame shifts arising from the cloning procedure.

The amino-acid translation of the resulting 6×His-Fes-CR-FLAG nucleotide sequence yielded a polypeptide consisting of 281 residues and with an estimated molecular weight of 31 929.30 Da as calculated using the protein-analysis tools of the *ExPASy* server (Gasteiger *et al.*, 2003): HHHHHHLNHEDLVLGEQIGRGNFGEVFSGRLRADNTLVAVKSCRETLPPDLKAKFLQEARILKQYSHPNIVRLIGVCTQKQPIYIVMELVQGGDFLTFLRTEGARLRVKTLLQMVGDAAAGMEYLESKCCIHRDLAARNCLVTEKNVLKISDFGMSREEADGVYAASGGSRQVPVKWTAPEALNYGRYSSESDVWSFGILLWETFSLGASPYPNLSNQQTREFVEKGGRLPCPELCPDAVFRLMEQCWAYEPGQRPSFSTIYQELQSIRKRHRDYKDDDDK.

By following the QIAexpressionist protocol (Qiagen), which is designed for high-level expression of exogenous proteins, the pQE-6×His-Fes-CR-FLAG plasmid was then used to transform the *Escherichia coli* strain M15pREP4 provided with the kit.

E. coli M15pREP4 bacteria harbouring the recombinant plasmid were grown in 1 l medium containing 16 g tryptone B, 10 g yeast extract B, 5 g NaCl (2×TY broth, Q-Biogene) supplemented with 100 µg ml^{-1} ampicillin at 310 K to an absorbance of 0.7 at 600 nm. Protein expression was induced at 303 K by the addition of isopropyl 1-thio-β-D-galactopyranoside (IPTG; Applichem) to a final concentration of 1 m*M*. After 4 h, the bacteria were collected by centrifugation and resuspended in 50 ml phosphate-buffered saline with buffering capacity ∼8 m*M* (PBS; 1.47 m*M* potassium phosphate monobasic, 8.1 m*M* sodium phosphate dibasic, 2.68 m*M* potassium chloride, 137 m*M* sodium chloride pH 7.4), 0.1 m*M* phenylmethanesulfonyl fluoride (PMSF, Sigma). The cells were sonicated on ice (4 × 60 s, with 60 s intervals) and the suspension was centrifuged at 12 000*g* for 30 min. The supernatant was loaded onto a 30 ml Ni Sepharose column (Amersham) pre-equilibrated with PBS. The column was washed with 300 ml PBS at a flow rate of 5.0 ml min^{-1} and the sample was eluted using a 0–500 m*M* gradient of imidazole (Sigma) in PBS over 125 ml. 20 µl aliquots of each eluted fraction were run on SDS–PAGE. The fractions corresponding to the major peak of purified 6×His-Fes-CR-FLAG (eluting at ∼350 m*M* imidazole) were pooled. The protein concentration was determined by microBCA (Pierce) using a 2 mg ml^{-1} bovine serum albumin solution as a standard. The purification yield was 5.5 mg per litre of culture.

The protein was concentrated to a final volume of 0.55 ml at 10 mg ml^{-1} using a 10 kDa cutoff centrifugal concentrator (Millipore). Imidazole was then removed by diafiltration. In order to perform this, the sample was first diluted with 15 ml PBS (∼30 sample volumes) and then concentrated to 0.5 ml using a 10 kDa cutoff centrifugal concentrator (Millipore). This step was repeated three times, leading to essentially complete removal of imidazole. 2 µl of the resulting concentrated sample was analysed by denaturing SDS–PAGE. The concentrated protein in PBS buffer was then used for subsequent crystallization experiments.

3. Protein crystallization and preliminary diffraction data analysis

Purified 6×His-Fes-CR-FLAG was used in an extensive search for suitable crystal-growth conditions using sparse-matrix crystallization kits (Hampton Research). Hanging drops were prepared in Cryschem 24-well plates (Hampton Research) by mixing 2 µl protein solution (10 mg ml^{-1} in PBS) with 1 µl reservoir solution and were equilibrated against 400 µl reservoir solution. Condition No. 20, which contained 1.6 *M* magnesium sulfate and 0.1 *M* MES pH 6.5, led to crystal growth in two weeks. After optimization of the physicochemical parameters affecting crystallization, the best conditions were obtained at 300 K using the hanging-drop vapour-diffusion

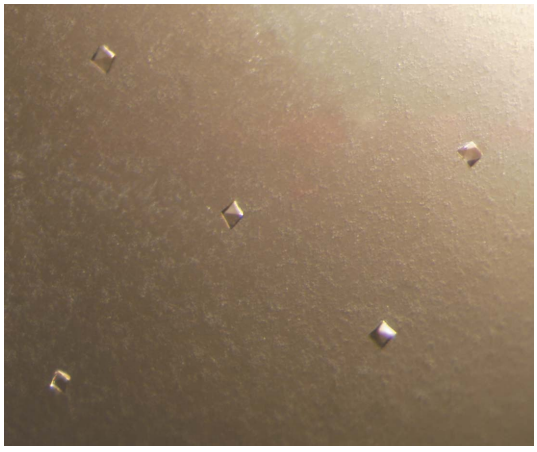

Figure 1
Crystals (dimensions about 0.05 × 0.02 × 0.02 mm) of the catalytic domain of the nonreceptor tyrosine kinase Fes.

method. A droplet prepared as described above was equilibrated against a crystallization well containing 400 µl reservoir solution (1.6 *M* $MgSO_4$, 0.1 *M* MES pH 6.5). Specifically, the protein solution was prepared by diluting the protein to 5 mg ml^{-1} with PBS and mixing 99 µl of this sample with 1 µl of the ATP analogue 5′-adenylylimido-diphosphate (AMP-PNP) to give a final 10:1 AMP-PNP:protein molar ratio.

Under these conditions, bipyramidal crystals (0.05 × 0.02 × 0.02 mm) grew after 15 d (Fig. 1). X-ray diffraction data were collected on a MAR Mosaic 225 CCD detector at 100 K and at a wavelength of 0.8726 Å at the European Synchrotron Radiation Facility (ESRF, Grenoble, France; beamline ID23-2). Determination of cryoprotection conditions led to the observation that the crystallization solution was sufficient to prevent ice formation on the sample, showing that 1.6 *M* $MgSO_4$ was sufficient as a cryoprotectant. Crystals were mounted on 0.05 mm cryoloops (Hampton Research) and survived for 900 s in the X-ray beam. They belonged to space group *F*432, with unit-cell parameters $a = b = c = 220.47$ Å. Diffraction data were collected to a resolution of 3.60 Å and were scaled to 3.90 Å resolution. Estimation of the V_M packing number (3.50 $Å^3$ Da^{-1}) suggested the presence of one molecule in the asymmetric unit, with a solvent content of 64% (Matthews, 1968). X-ray diffraction data were integrated with the program *MOSFLM* v.6.2.3 (Leslie, 1992) and scaled with *SCALA* v.3.2.15 (Evans, 1997). The relevant statistics of the data collection are reported in Table 1. Three-dimensional structure solution using molecular replacement and the structure of the kinase domain of Abelson tyrosine kinase as a model (Schindler *et al.*, 2000; PDB code 1fpu) is in progress. This structure was chosen as a molecular-replacement probe because of its high sequence identity (42%) and similarity (64%) to the Fes-CR domain as determined by *BLAST* (Henikoff, 1996). Crystallization screenings to obtain better diffracting crystals are under way in parallel for both the apoprotein and its complexes with inhibitors.

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Table 1
Data-collection statistics.

Values in parentheses are for the last shell.

Beamline	ESRF ID23-2
Wavelength (Å)	0.8726
Resolution range (Å)	127.00–3.90 (4.11–3.90)
Total No. of reflections collected	49514
Unique reflections	4568
Redundancy	10.8
Completeness (%)	100 (100)
R_{sym} (%)	21.7 (59.0)
$\langle I/\sigma(I)\rangle$	10.3 (3.1)
Wilson plot *B* factor ($Å^2$)	32.9

† $R_{sym} = \sum_{\mathbf{h}}\sum_{l}|I_{\mathbf{h}l} - \langle I_{\mathbf{h}}\rangle|/\sum_{\mathbf{h}}\sum_{l}\langle I_{\mathbf{h}}\rangle$, where I_l is the *l*th observation of reflection **h** and $\langle I_{\mathbf{h}}\rangle$ is the weighted average intensity for all observations *l* of reflection **h**.

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