Over-Expression, Detergent Extraction, Purification, and Preliminary Analysis of Recombinant FERROPORTIN from Various Vertebrate Species

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INTRODUCTION

This paper focuses on the over-expression, detergent-extraction and purification of human, mouse, and zebrafish forms of ferroportin (Fpn) for use in various biophysical analyses and crystallization attempts. Prior to our studies, recombinant Fpn expression had been limited to small-scale eukaryotic systems. For example, the initial studies in which Fpn was identified and partially characterized involved small-scale recombinant expression Fpn utilizing such methods as microinjection in Xenopus oocytes (Donovan et al. 2000; McKie et al. 2000) or zebrafish embryos (Donovan et al. 2000), or by transient transfection in mammalian tissue culture lines, such as Madin-Darby canine kidney cells (MDCK) (McKie et al. 2000), CaCo-2 cells (McKie et al. 2000), or HEK293T cells (Abboud and Haile 2000). Fpn expression in these systems was evaluated by sensitive detection techniques such as Western blotting, direct fluorescence (in the case of Fpn- GFP), or immunofluorescence. Subsequent Fpn studies used these same small-scale eukaryotic expression methods (De Domenico et al. 2005, 2006, 2007a,b,c;

Drakesmith et al. 2005; Goncalves et al. 2006; Nemeth et al. 2004; Schimanski et al. 2005; 2008), and no attempts at Fpn over-expression or purification beyond those reported in chapter 2 (Rice et al. 2009) have been reported to date. Our Fpn over-expression strategy involved both prokaryotic and eukaryotic methods. Prokaryotic expression tests were performed in various strains of the bacterium E. coli and eukaryotic expression tests were performed using baculoviruses to infect High Five and Sf9 insect cell lines. Initial efforts in both prokaryotic and eukaryotic systems yielded no Fpn expression (as assessed by Western blotting), however after extensive testing of various Fpn constructs under a large number of expression conditions we were able to successfully over-express human, mouse, and zebrafish Fpns at adequate levels for biophysical characterization and crystallization trials as will be described in this chapter. Once an over-expression protocol was worked out, detergent-extraction protocols were developed for the solubilization of Fpn from prokaryotic and eukaryotic membranes. We found that Fpns expressed in insect cell plasma membranes were much more amenable to detergent extraction than Fpns expressed in the inner membrane of E. coli. The detergents that were capable of extracting Fpn were then used to develop purification protocols. The eukaryotic expression and purification was reported in Chapter 2 (Rice et al. 2009), but due to word limits in the published format, certain details were left out or not covered in full. These details will be discussed in more detail in this chapter. The first section of this chapter reports the expression, extraction, and purification for the expression of Fpn in bacteria, and the second portion is devoted to similar studies in insect cells.

BACTERIAL EXPRESSION CONSTRUCTS

Human and mouse Fpn genes were gifts from Professor Alain Townsend (Oxford, UK) and zebrafish Fpn gene was a gift from Professor Nancy Andrews (Duke University). The human gene was contained within a mammalian expression construct, with the name hfpn1-V571A-myc-His6-pcDNA3.1. It contained an accidentally introduced V571A mutation. This mutation was reversed by site-directed mutagenesis, before sub-cloning of this gene continued. The mouse gene was contained within a mammalian expression construct, with the name mfpn1-

myc-His6-pcDNA3.1. The zebrafish gene was contained within a mammalian expression construct with the name zfpn1-GFP-pEGFP-N1. The human and zebrafish genes naturally contained an NdeI restriction enzyme sequence within their ORFs, and these were removed by mutagenesis prior to further mutagenesis and/or sub-cloning into bacterial expression vectors. Our cloning strategy for bacterial expression constructs was to work with three separate forms of each Fpn: wild-type; A77D; and N144H. The A77D and N144H mutants have been identified as diseasecausing Fpn mutations (Montosi et al. 2001; Njajou et al. 2001), however, how these mutations lead to disease was unknown. We surmised that these mutants were worth pursuing alongside the wild-type Fpn for initial expression studies, particularly if expression of the fully functional wild-type Fpn was toxic due to unregulated iron export from cells. Each Fpn form was then directionally subcloned into the NdeI and NotI sites of pET-23a (Novagen) for a C- terminal 6× His tag, pET-28b (Novagen) for an N-terminal 6× His tag, pMAL-p2E (New England Biolabs) for an N-terminal maltose binding protein fusion, or alternately into pET-28b adding His and/or StrepTagII tags via PCR. Gene expression in these vectors was driven by the T7 promoter in pET vectors and by Ptac promoter in the pMAL vector, both of which were induced by isopropyl β-D-

1- thiogalactopyranoside (IPTG) addition. Sequencing the constructs revealed an unplanned result for two forms of human Fpn in pET-28b (wild-type and N144H), in which the final constructs had both N- and C-terminal His tags. These constructs (pAER113a and pAER115a) with affinity tags on both termini were included in the expression screening.

The bacterial expression constructs are summarized in table below.

Bacterial expression constructs **Plasmid Name Gene or Insert Name**

PARENT VECTOR N-TERMINAL TAG C-TERMINAL TAG

pAER112 human Fpn (w.t.) pET-23a none His6
pAER113a human Fpn (w.t.) pET-28b His6 His6
pAER114 human Fpn N144H pET-23a none His6
pAER115a human Fpn N144H pET-28b His6 myc-His6
pAER116 human Fpn A77D pET-23a none His6
pAER117 human Fpn A77D pET-28b His6 none
pAER118 mouse Fpn (w.t.) pET-23a none His6
pAER119 mouse Fpn (w.t.) pET-28b His6 none
pAER120 mouse Fpn N144H pET-23a none His6
pAER121 mouse Fpn N144H pET-28b His6 none
pAER122 mouse Fpn A77D pET-23a none His6
pAER123 mouse Fpn A77D pET-28b His6 none
pAER124 zebrafish Fpn (w.t.) pET-23a none His6
pAER125 zebrafish Fpn (w.t.) pET-28b His6 none
pAER126 zebrafish Fpn N144H pET-23a none His6
pAER128 zebrafish Fpn A77D pET-23a none His6
pAER136 mouse Fpn (w.t.) pMAL-p2E MBP His6
pDZS110 human Fpn (w.t.) pET-28b His6 Strep
pET-HS-1c human Fpn (w.t.) pET-28b His6-Strep none

pET-HS-3a human Fpn (w.t.) pET-28b His6-Strep His6
Small-Scale Tests of Fpn Expression in E. coli

Expression constructs were tested for Fpn expression in over 900 expression conditions, exploring variables such as: E. coli strain; induction temperature; growth media formulation; induction length; concentration of inducer; and aeration levels. Media formulations are summarized in table 3.2 and expression tests are summarized in table 3.3. Overnight cultures in the media of choice were grown from glycerol stocks at 37 ºC shaking at 240 rpm. The following morning, 5 mL cultures in 50 mL plastic conical vials (Falcon) with their caps loosely taped in place to allow adequate aeration were inoculated with 100 µL of overnight culture and incubated at 37 ºC and 240 rpm. The optical density, as measured at 600 nm (OD600), of the growth culture was monitored routinely.

When cultures reached an OD600 between 0.5 and 1.0, cultures were moved to an incubator at a preset temperature agitating at 240 rpm, a 1 mL fraction was removed as the zero time point, and the remaining 4 mL were induced by addition of IPTG to a final concentration of 0.4 mM IPTG. Induced cultures were allowed to incubate for various times before 1 mL fractions were removed for analysis. Cells from fractions removed for analysis were spun down for 1 min at 13,000×g, after which the supernatant was aspirated and the cell pellet frozen at −20 ºC until Western blot analysis could be performed, as described below. Using this technique, a single 5 mL culture would be used to test up to 4 time points for a given condition. The expression tests summarized in table 3.3 that report >4 time points were performed in larger volumes but otherwise adhered to the protocol described here.

GROWTH MEDIA FORMULATIONS

LB 2×YT SOC TB TB1 TB2 TB3 TB4 TB5 TB6 TB7
bactotryptone
(g/L) 10 16 20 12 12 12 12 12 12 12 12
yeast extract
(g/L) 5 10 5 24 24 12 - 8 12 - -
malt extract (g/L) - - - - - - - 8 12 24 12
beef extract (g/L) - - - - - 12 24 8 - - 12
NaCl (g/L) 10 5 0.58 - - - - - - - -
KCl (g/L) - - 0.18 - - - - - - - -
MgCl2•6H20 (g/L) - - 2.03 - - - - - - - -
MgSO4 (g/L) - - 1.20 - - - - - - - -
1M glucose
(mL/L) - - 20 - - - - - - - -
gycerol (mL/L) - - - 4 8 8 8 8 8 8 8
KH2PO4 (g/L) - - - 2.31 2.31 2.31 2.31 2.31 2.31 2.31 2.31
K2HPO4 (g/L) - - - 12.54 12.54 12.54 12.54 12.54 12.54 12.54 12.54

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