ACTA ABP BIOCHIMICA POLONICA

Vol. 59, No 4/2012
685–691
on-line at: www.actabp.pl

Regular paper

Bacterially expressed truncated F2 domain of *Plasmodium falciparum* EBA-140 antigen can bind to human erythrocytes*

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The recently identified erythrocyte binding antigen-140 (EBA-140) is a member of the *Plasmodium falciparum* DBL family of erythrocyte binding proteins, which are considered as prospective candidates for malaria vaccine development. The EBA-140 ligand is a paralogue of the well-characterized *P. falciparum* EBA-175 antigen. They share homology of domain structure, including Region II, which consists of two homologous F1 and F2 domains and is responsible for ligand-erythrocyte interaction during invasion. It was shown that the F2 domain of EBA-175 antigen seems to be more important for erythrocyte binding. In order to study activity and immunogenicity of EBA-140 antigen F2 domain, it is necessary to obtain recombinant protein of high purity and in a sufficient amount, which used to pose a challenge due to the high content of disulphide bridges. Here, we present a new method for expression and purification of *Plasmodium falciparum* EBA-140 antigen F2 domain in *E. coli* Rosetta-gami strain in fusion with the maltose binding protein (MBP). The truncated F2 domain formed by spontaneous proteolytic degradation of the fusion protein was purified by affinity chromatography on Ni-NTA resin followed by size exclusion chromatography. Molecular mass of this protein was confirmed by mass spectrometry. Its N-terminal amino acid sequencing revealed a proteolytic cleavage site within the F2 domain. The proper folding of the recombinant, truncated F2 domain of EBA-140 antigen was confirmed by circular dichroism analysis. The truncated F2 domain can specifically bind to human erythrocytes but its binding is not as efficient as that of full Region II. This confirms that both the F1 and F2 domains of EBA-140 antigen are required for effective erythrocyte binding.

Key words: *Plasmodium falciparum,* EBA-140 antigen, recombinant F2 domain expression, truncated F2 domain purification, human erythrocyte binding

Received: 30 October, 2012; revised: 25 November, 2012; accepted: 05 December, 2012; available on-line: 11 December, 2012

INTRODUCTION

Malaria is caused in humans by five species of *Plasmodium*: *P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi* and *P. falciparum*. Although *P. vivax* is most widely spread, *P. falciparum* is responsible for the greatest number of deaths (Greenwood *et al.*, 2008; Pierce & Miller, 2009). Erythrocyte invasion by the blood-stage malaria parasites is a multistep process involving specific interactions between ligands of merozoites and receptors on the red blood cells (Gaur *et al.*, 2004; Cowman & Crabb, 2006; Jaśkiewicz *et al.,* 2010). Two protein families play central roles in this process: the Duffy binding-like (DBL) and the reticulocyte binding-like (RBL) proteins. In *P. falciparum* there are several members of the DBL family, including erythrocyte binding-like (EBL) proteins, which enable the parasite to interact with independent receptors and define alternative invasion pathways. Four functional *P. falciparum* EBL proteins have been characterized: erythrocyte binding antigen-175 (EBA-175), EBA-140 (also known as BAEBL), EBA-181 (also known as JESEBL) and erythrocyte binding ligand-1 (EBL-1) (Cowman & Crabb, 2006). All these proteins contain several conserved regions, including Region II, which is involved in receptor binding and consists of two cysteine-rich domains: F1 and F2. EBA-175 is a well-studied *P. falciparum* merozoite ligand that binds to glycophorin A (GPA) on human erythrocytes (Sim *et al.* 1994; Pandley *et al.*, 2002; Tolia *et al.* 2005; Jaśkiewicz, 2007). The recently identified EBA-140 homologous ligand binds to glycophorin C (GPC), a minor erythrocyte sialoglycoprotein (Jaśkiewicz, 1991), and mediates a distinct invasion pathway of human erythrocytes (Thompson *et al.*, 2001; Narum *et al.*, 2002; Gilberger *et al.*, 2003; Lobo *et al.*, 2003; Maier *et al.*, 2003).

The major limitation in studies on the specificity and immunogenicity of EBA ligands is their expression and purification as soluble and properly folded recombinant proteins in sufficient amounts. Recombinant Region II (F1 and F2 domains) or F2 domain of EBA-175 ligand were obtained using bacterial (Pandley *et al.,* 2002), insect (Liang *et al.,* 2000; Ockenhouse *et al.,* 2001) or yeast cells (Zhang & Pan, 2005). Recombinant F2 domain of EBA-175 antigen expressed in *E. coli* was purified from inclusion bodies and renatured by oxidative refolding. In order to increase the level of EBA-175 F2 domain expression, sequences coding for the F2 domain, based on *E. coli* and *Pichia* codon usage, were designed (Yadava & Ockenhouse, 2003). The protein expressed in *P. pastoris* was soluble and functional in comparison to the insoluble F2 domain produced in *E. coli* as inclusion bodies. Taking into account that EBA-175 Region II contains several cysteine-rich motifs, the eukaryotic yeast or baculovirus expression system seemed to be most suitable.

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*Preliminary report: an abstract on 22nd IUMB&37th FEBS Congress, 4-9 September, 2012 Seville, Spain.

Abbreviations: EBA, erythrocyte binding antigen; GdmCl, guanidine hydrochloride; GPA, glycophorin A; GPC, Glycophorin C; IPTG, isopropyl β-D-1-thiogalactopyranoside; MBP, maltose binding protein; RBC, red blood cells..

Regarding expression of the EBA-140 ligand, only two reports are available to date. Similarly to EBA-175 antigen, EBA-140 binding Region II was obtained in the baculovirus expression system (Kobayashi *et al*., 2010) or bacterial expression system (Lin *et al*., 2012). Recombinant Region II and F1, F2 domains were also expressed separately on the surface of Chinese hamster ovary (CHO-K1) (Jiang *et al*., 2009), COS7 (Mayer *et al*., 2002) and HEK- 293T cells (Lin *et al*., 2012).

It was shown that mouse and rabbit antibodies against the F2 domain of EBA-140 antigen inhibit the EBA-140/GPC invasion pathway of human erythrocytes by *P. falciparum* (Narum *et al*., 2002; Maier *et al*., 2003). There is also one study on the naturally acquired antibody response to the EBA-140 ligand in populations of individuals living in endemic areas of Brazil (Ford *et al*., 2007). These modest data could suggest the potential importance of EBA-140 antigen as a novel vaccine candidate. This has encouraged us to obtain the recombinant EBA-140 ligand binding region in order to study its activity and immunogenicity. We have chosen the bacterial expression system as the most convenient and inexpensive method to obtain recombinant proteins in sufficient amounts. This report describes bacterial expression and purification of the truncated F2 domain of *P. falciparum* EBA-140 antigen. We have obtained pure and correctly folded functional recombinant protein; containing 197 of the 335 amino acid residues of the full-length F2 domain. This truncated form of EBA-140 F2 domain was shown to bind specifically to human erythrocytes but its binding was not as efficient as that of the whole Region II. This indicates that both DBL domains of EBA-140 antigen are required for effective binding to erythrocytes as shown in previous reports (Mayer *et al*., 2002; Lin *et al*., 2012).

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* XL1 Blue cells (Stratagene, La Jolla, CA, USA) for plasmid cloning and Rosetta-gami (Novagen-Merck, Darmstadt, Germany) for protein expression were used. Cloning vector pDrive (Qiagen, Hilden, Germany) and bacterial expression vector pMALc2x (New England BioLabs, Ipswich, MA, USA) were used.

Cloning of EBA-140 antigen Region II. Region II (amino acids 141–756) of the EBA-140 ligand (GenBank: AF 332918_1) was cloned from genomic DNA of *P. falciparum* clone Dd2 (ATCC, MR4, Manassas, VA, USA) with forward primer: CAA TAT ACG TTT ATA CAG AAA CGT ACT CAT TTG TTT GCT and reverse primer: TAT ATC GTG TTT TGT TTT AGG ATA TTT A. Taq DNA Polymerase (Fermentas, Vilnius, Lithuania) was used in 35 cycles of amplification (94°C, 30 s; 54°C, 30 s; 72°C, 2 min 30 s) after a hot start at 94°C for 5 min and 10 min of final extension at 72°C. The PCR product was purified using a gel extraction kit (Qiagen) and cloned into pDrive cloning vector (Qiagen) using T4 DNA ligase (Fermentas). The obtained pDrive-RII recombinant plasmid was transformed into *E. coli* XL1 Blue competent cells and selected on LB-agar with 100 μg/ml ampicillin (Polfa Tarchomin, Warszawa, Poland). The sequence was confirmed by restriction fragment analysis and DNA sequencing.

Construction of pMalc2x-F2 expression vector. The recombinant plasmid pDrive-RII encoding Region II of the EBA-140 ligand was used as a template to amplify the F2 domain (amino acids 422–756) with AccuPrime Pfx SuperMix (Invitrogen, Carlsbad, CA, USA) in 35 cycles of amplification (95°C, 15 s; 54° C, 30 s; 68°C, 90 s) after a hot start at 95°C for 5 min and 10 min of final extension at 68°C. Forward primer: CGC GGG TCT AGA AGA TAT ACT GCT ACT ATT ATT AAA AGT introducing the XbaI restriction site and reverse primer: CGA TAT AAG CTT CTA ATG ATG ATG ATG ATG ATG CAG ATC CTC TTC TGA GAT GAG TTT TTG TTC TAT ATC GTG TTT TGT TTT AGG introducing the Hind III restriction site and c-myc and 6xHis tags were used. The PCR product was purified using a PCR purification kit (Qiagen) and digested with restriction enzymes XbaI and HindIII (Fermentas). The digested and purified product was cloned into pMalc2x vector using T4 DNA ligase (Fermentas) transformed into *E. coli* XL1 Blue competent cells and selected on LB-agar with 100 μg/ml ampicillin. The pMalc2x-F2 plasmid was transformed into *E. coli* Rosetta-gami competent cells in order to express recombinant fusion protein.

Expression of MBP-F2 fusion protein in *E. coli*. Recombinant F2 domain of EBA-140 antigen was expressed in *E. coli* Rosetta-gami as the fusion protein with maltose binding protein (MBP) at the N-terminal end and with c-myc and 6xHis tags at the C-terminus. A single bacterial colony containing pMalc2x-F2 vector encoding the MBP-F2 protein was inoculated into 10 ml LB medium supplemented with 100 μg/ml ampicillin and 30 μg/ml chloramphenicol (Roth, Karlsruhe, Germany). After overnight cultivation (30°C, 190 rpm) 2 ml aliquots were transferred into 200 ml fresh LB medium containing 100 μg/ml ampicillin. The bacterial culture was continued (30°C, 180 rpm) until OD_{600nm} reached 0.8. To induce the MBP-F2 fusion protein expression, bacterial cells were cultured with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Roth) at 30°C or 16°C for 20 h. In order to assess the influence of cultivation time on protein expression level, small culture samples were taken 2, 4, 6, 14, 16, 18 and 20 h after induction and disintegrated by sonication. Protein content was evaluated by polyacrylamide gel electrophoresis (SDS/PAGE). These initial studies indicated that optimal expression of the MBP-F2 fusion protein was achieved in *E. coli* Rosetta-gami cells cultivated with 0.3 mM IPTG at 30°C for 4 h. Large-scale expression of the MBP-F2 fusion protein was performed in 3 liter culture of *E. coli* Rosetta-gami in conditions described above. The bacteria were then harvested by centrifugation at 6000 rpm at 4°C and stored at –20°C.

Purification of MBP-F2 fusion protein. The *E. coli* cells harvested by centrifugation were resuspended in pre-cooled column buffer (50 mM Tris/HCl, 300 mM NaCl, pH 7.5) containing 1 mM EDTA, 5 mM β-mercaptoethanol, 1 mM PMSF and protease inhibitor cocktail (10 μl per 1 ml of buffer, Sigma-Aldrich, St. Louis, MO, USA). The suspension was frozen in dry ice-ethanol bath, stored overnight at –20°C and then thawed in cold water. The cells in the suspension were lysed by sonication and centrifuged at 14 000 rpm for 30 min at 4°C. The supernatant was added to 10 ml of nickel nitrilotriacetic acid resin (Ni-NTA, Qiagen) equilibrated with the column buffer and then the whole suspension was mixed for 3 h at 4°C. The resin was loaded onto a 50 ml column washed with 2 l of the column buffer. To remove nonspecifically binding proteins 20 ml of the column buffer containing 20 mM imidazole was used. The bound proteins were subsequently eluted with the column buffer containing imidazole at the following concentrations: 50 mM, 150 mM and 200 mM. Fractions

were analyzed by SDS/PAGE and Western blotting. Fractions eluted with 200 mM imidazole containing the MBP-F2 fusion protein and truncated F2 domain were collected.

Proteins were separated by SDS/PAGE according to the method of Laemmli (Laemmli, 1970) using 10% gel and visualized with Coomassie Brilliant Blue R-250 (CBB, Merck). The PageRuler Prestained Protein Ladder (Fermentas) was used as protein standards.

Western blotting. Proteins fractionated by SDS-PAGE were transferred to nitrocellulose membrane (Schleicher & Schuel, Dassel, Germany) according to the method of Towbin (Towbin *et al*., 1979) and detected with mouse monoclonal antibodies (MoAb) directed against c-myc epitope (9E10, ATCC) and histidine-tag (His-1, Sigma-Aldrich).

Analytical size-exclusion chromatography. Fractions containing the MBP-F2 protein and truncated F2 domain, eluted with 200 mM imidazole, were pooled and concentrated ten times using a 10 000 M_r cut-off Amicon Ultra device (Millipore, Billerica, MA, USA). A sample containing about 750 μg of total protein was analyzed on an analytical 10/300 GL Superdex 200 column (AKTA Purifier, GE Healthcare, Uppsala, Sweden) in the column buffer. Protein content was evaluated spectrophotometrically at 280 nm. In order to optimize separation of high molecular mass aggregates containing the MBP-F2 fusion protein and truncated F2 domain, samples were analyzed in the column buffer containing different concentrations of DTT (5–25 mM) and guanidinium chloride (0.2–0.75 M, GdmCl).

Analytical size-exclusion chromatography used for evaluation of the purified, truncated F2 domain (250 μg) was performed on a 10/300 GL Superdex 200 column equilibrated with the column buffer containing 10% glycerin at a flow rate of 0.4 ml/min at room temperature (RT). Protein molecular weight standards used were: 66.4 kDa albumin, 43 kDa ovalbumin, 25 kDa chymotrypsinogen A, 14.4 kDa cytochrome c (Sigma-Aldrich).

Preparative size-exclusion chromatography of truncated F2 domain. The concentrated sample containing about 7 mg of total protein was mixed in volume ratio 1:2 with the column buffer containing 0.2 M GdmCl and 10 mM DTT, incubated for 1 h at 25°C and loaded onto a preparative Hiload 26/60 Superdex 200 size-exclusion column (AKTA Purifier), previously equilibrated with the column buffer, and eluted at a flow rate of 1 ml/min at RT. The eluted fractions were analyzed by SDS/PAGE. Fractions containing the purified, truncated F2 domain were pooled and concentrated using a 10 000 M_r cut-off Amicon Ultra device to 0.5 mg/ml of protein and then dialyzed at 4°C for 20 h in the three following steps: against 50%, 20% and finally 10% glycerin in the column buffer. Protein concentration was determined spectrophotometrically at 280 nm by the Bradford method (Bradford, 1976) using a Nanoquant protein assay reagent (Roth).

Circular dichroism of purified, truncated F2 domain. The folding state of the purified, truncated F2 domain (at 2 μM concentration) and of the whole baculovirus-expressed Region II of EBA-140 antigen (GenScript, Hong Kong) were evaluated by circular dichroism (CD) spectroscopy. CD spectra were recorded on a Jasco J-600 spectropolarimeter (Jasco Inc., Easton, MD, USA)), at RT. Path length of 1 mm was used. Each spectrum represents the average of four scans. The data are presented as mean residue molar ellipticity [θr].

Mass spectrometry. Molecular weight of the truncated F2 domain was confirmed by MALDI-TOF MS (matrix-assisted laser desorption/ionization-time of flight mass spectrometry) using an Autoflex III mass spectrometer (Bruker Daltonics, Hamburg, Germany). 10 μl of protein sample (0.5 mg/ml) in 50 mM Tris/HCl, 300 mM NaCl, 10% glycerin were desalted using reversed-phase zip-tips C-18 (Millipore) according to the manufacturer's recommendations. The bound protein was eluted with 2 μl of 0.05% trifluoroacetic acid in 50% acetonitrile. The obtained sample was mixed with a matrix solution. The mixtures of protein sample and matrix solution were loaded onto the MALDI target and dried. The mass spectra were scanned in the range of 8000 + 70 000 m/z. The external calibration was carried out using Protein Standard II (Bruker Daltonics): trypsinogen, protein A, and bovine albumin.

Determination of three N-terminal amino acids of the truncated F2 domain fragment was carried out by Edman degradation by BioCentrum (Krakow, Poland).

Flow cytometry analysis. 50 μl of the truncated F2 domain (0.5 mg/ml) or of the whole Region II (0.15 mg/ml) - were incubated with 3×10^5 native human red blood cells (RBC) treated with trypsin (Sigma-Aldrich) or neuraminidase (*Vibrio cholerae*, Serva, Heidelberg, Germany), in phosphate buffered saline (PBS), pH 7.4, for 2 h at 4°C. After three washes with PBS, RBC were incubated for 1 h at 4°C with rabbit serum (diluted 1:200) raised against the whole Region II. Then, after 3 washes with PBS, RBC were incubated for 30 min at 4°C with FITC-conjugated swine anti-rabbit Ig antibody (Dako-Cytomation, Glostrup, Denmark). Erythrocytes were analyzed for fluorescence intensity using flow cytometry (FACSCalibur, BD Biosciences, San Jose, Ca, USA).

Immunization of rabbits. Rabbits were immunized with 50 μg of baculovirus-expressed Region II (GenScript) in MPL adjuvant (Baldridge & Crane, 1999) and boosted in three-week intervals with 50 μg of the antigen. One rabbit was immunized with MPL adjuvant alone to provide control serum. Sera were collected 14 days after the fourth immunization and used for assays.

RESULTS AND DISCUSSION

Expression of F2 domain of EBA-140 antigen

Recombinant F2 domain was expressed in *E. coli* Rosetta-gami cytoplasm as a fusion protein with MBP. It is known that MBP can promote proper folding of fusion proteins and thus increase their solubility. It was frequently observed that MBP fusion proteins could be efficiently overexpressed and exhibited enhanced solubility and stability compared to their untagged counterparts (Kapust & Waugh, 1999). *E. coli* Rosetta-gami is a trxB mutant, which facilitates cytoplasmic disulfide bond formation (Stewart *et al*., 1998) and can be particularly useful for the folding of proteins containing several disulphide bridges, like the F2 domain of *P. falciparum* EBA-140 antigen.

In order to obtain soluble recombinant F2 domain of the EBA-140 ligand in a sufficient amount, pilot experiments were performed to determine optimal cultivation conditions, including appropriate growth temperature and time of harvest after induction. Initial studies suggested that optimal expression of MBP-F2 fusion protein was achieved in *E. coli* Rosetta-gami cells induced with 0.3 mM IPTG and cultivated at 30°C for 4 h or at 16°C for 14 h (Fig. 1). However, the recombinant MBP-F2 fusion protein was shown to be rapidly degraded: in addition to the MBP-F2 protein (~ 85 kDa), a shorter

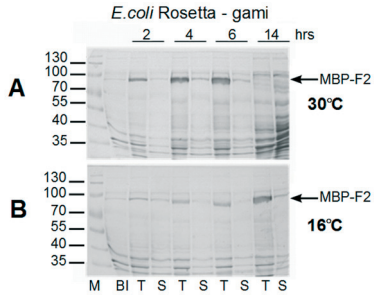

Figure 1. Optimization of MBP-F2 fusion protein expression in *E. coli* strain Rosetta-gami at 30°C (A) and 16°C (B) and various cultivation times after 0.3 mM IPTG induction.
SDS/PAGE of bacterial proteins in 10% gel stained with CBB; T, total extract; S, soluble fraction; BI, total extract before expression induction; M, protein molecular weight standards.

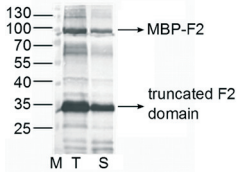

Figure 2. Spontaneous degradation of MBP-F2 fusion protein in the cytoplasm of *E. coli* Rosetta-gami cells.
Western blotting with anti-myc MoAb; T, total extract; S, soluble fraction; M, protein molecular weight standards; MBP-F2 fusion protein (~ 85 kDa); truncated F2 domain (~ 30 kDa).

protein: truncated F2 domain (F2 domain fragment, ~30 kDa) was present in cell lysate (Fig. 2). Modifications of growth conditions did not significantly reduce this degradation. These results suggest that the recombinant MBP-F2 fusion protein may be susceptible to spontaneous degradation in the cytoplasm of *E. coli* cells. The large scale expression of the MBP-F2 fusion protein was performed in Rosetta-gami 3 l bacterial culture transformed with pMALc2x-F2 expression vector, induced with 0.3 mM IPTG and cultured at 30°C for 4 h.

Purification of EBA-140 truncated F2 domain

MBP-F2 fusion protein expressed in the conditions described above was purified by affinity chromatography on Ni-NTA resin. Fractions eluted with 200 mM imidazole containing the MBP-F2 fusion protein and truncated F2 domain (Fig. 3) were pooled, concentrated and analyzed on an analytical 10/300 GL Superdex 200 column (Fig. 4).

It was observed that the MBP-F2 fusion protein forms aggregates with the truncated F2 domain (Fig. 4A). The aggregates are probably formed during MBP-F2 expression in *E. coli* cells and dissociate under reducing and denaturing conditions of SDS/PAGE. It has been shown previously that aggregation of recombinant proteins can

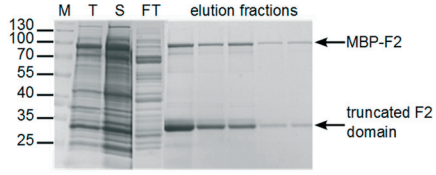

Figure 3. Affinity purification of EBA-140 truncated F2 domain on Ni-NTA resin.
SDS/PAGE of proteins in 10% gel stained with CBB; T, total extract; S, soluble fraction; FT, flow-through of Ni-NTA resin; M, protein molecular weight standards; MBP-F2 fusion protein (~ 85 kDa); truncated F2 domain (~ 30 kDa).

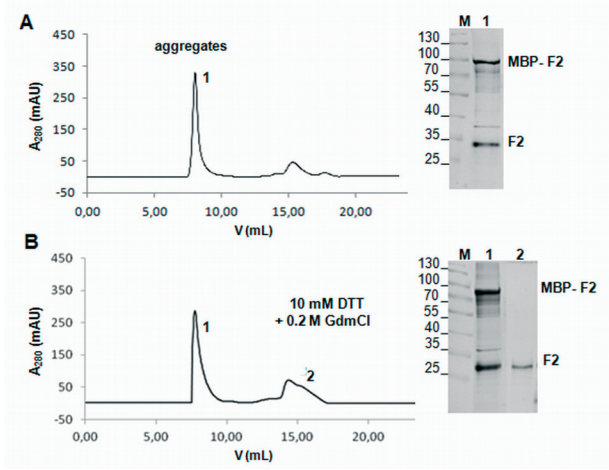

Figure 4. Analytical size-exclusion chromatography on a Superdex 200 column of affinity purified EBA-140 truncated F2 domain.
Chromatogram A (run in column buffer), peak 1 and SDS/PAGE lane 1, aggregates of MBP-F2 fusion protein with F2 domain fragment; Chromatogram B (run in column buffer containing 10 mM DTT and 0.2 M GdmCl), peak 1 and SDS/PAGE lane 1, aggregates of MBP-F2 fusion protein with the truncated F2 domain, peak 2 and lane 2, the truncated F2 domain; M, protein molecular weight standards.

occur during overexpression in bacterial cells because of high protein concentration (Villaverde & Carrió, 2003). Aggregation of a protein fused to MBP was also shown for N-terminal domains of the carcinoembryonic antigen (Czepczyńska-Krężel *et al.*, 2011). In the case of EBA-140 Region II (F1 and F2 domains) one of the causes of aggregation may be its natural ability to form dimers. It was shown before that recombinant EBA-175 homologous Region II exists in both monomeric and dimeric forms (Tolia *et al.*, 2005). The structures of EBA-175 antigen and its parologue EBA-140 are similar and therefore we speculate that the F2 domain of EBA-140 antigen may also be involved in domain to domain interaction.

Since the degradation product of the F2 domain accounted approximately for 50% of the obtained protein, we decided to purify this fragment. Therefore, it was necessary to find the optimal conditions for disrupting aggregates of the truncated F2 domain with the MBP-

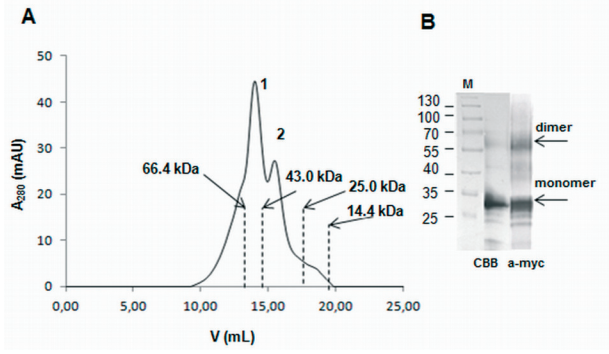

Figure 5. Analytical size exclusion chromatography on a Superdex 200 column of the pure EBA-140 truncated F2 domain (A).
Purified protein was analyzed by SDS/PAGE in 10% gel stained with CBB and Western blotting with anti-myc MoAb **(B)**; peak 1 corresponds to the dimer form of the truncated F2 domain and peak 2 corresponds to the monomer form of the truncated F2 domain; M, protein molecular weight standards; Elution volumes of standards used in column calibration are marked by dashed lines.

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  1 RYTATIIKSFLNGPAKNDVDIASQINVNDLRGFGCNYKSN
 41 NEKSWNCTGTFTNKFPGTCEPPRRQTLCLGRTYLLHRGHE
 81 EDYKEHLLGASIYEAQLLKYKYKEKDENALCSIIQNSYAD
121 LADIIKGSDIIKDYYGKKMEENLNKVNKDKKRNEESLKIF
161 REKWWDENKENVWKVMSAVLKNKETCKDYDKFQKIPQFLR
201 WFKEWGDDFCEKRKEKIYSFESFKVECKKKDCDENTCKNK
241 CSEYKKWIDLKKSEYEKQVDKYTKDKNKKMYDNIDEVKNK
281 EANVYLKEKSKECKDVNFDDKIFNESPNEYEDMCKKCDEI
321 KYLNEIKYPKTKHDIEQKLISEGDLHHHHHH
                    c-myc     6xHis
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Figure 6. The amino acid sequence of cloned *P. falciparum* EBA-140 antigen F2 domain (335 aa res.) with c-myc and 6xHis tags. The sequence of purified, truncated F2 domain (197 aa res.) is shaded in dark gray.

F2 fusion protein. Several reducing agents, pH values and chaotropic salts were tested and the dissociation of aggregates was monitored using the analytical Superdex 200 column. It was found that DTT at concentrations of 5–25 mM or GdmCl at concentrations of 0.2–0.75 M caused partial separation of the aggregates (data not shown). The best conditions for separation of the truncated F2 domain from aggregates were found to be 0.2 M GdmCl and 10 mM DTT used as components of the column buffer (Fig. 4B). However, we were unable to obtain complete disruption of the aggregates.

Purification of the truncated F2 domain was performed on the preparative Superdex 200 column in the conditions described in the Materials and Methods. It was noticed that 10–50% concentration of glycerin in the column buffer during dialysis of eluted fractions suppressed aggregate formation and increased solubility of the recombinant protein. The final yield of the truncated F2 domain was about 1.0 mg per liter of bacterial culture. The purified protein was stored in 50 mM Tris/HCl, 300 mM NaCl, 10% glycerin, pH 7.5.

Homogeneity of the purified, truncated F2 domain was confirmed by analytical size-exclusion chromatography (Fig. 5A). The Superdex 200 elution profile indicated two peaks: the first corresponding to the truncated F2 domain dimer (52.6 kDa, elution volume 14 ml) and the second one corresponding to the monomer of truncated F2 domain (26.3 kDa, elution volume 15.5 ml). This suggests that the truncated F2 domain may exist in solution in both monomer and dimer forms. Indeed, it was shown by SDS-PAGE and Western blotting that the truncated F2 domain migrated as a monomer (~ 26 kDa) and as a band corresponding to the dimer form (~52 kDa) (Fig. 5B). The dimerization is probably caused by hydrophobic interaction and salt bridges, as it was shown for Region II of *P. falciparum* EBA-175 ligand (Tolia *et al.*, 2005). The obtained truncated, recombinant F2 domain of EBA-140 antigen was more than 95% pure.

Molecular mass analysis of truncated F2 domain

Sequencing of the purified, truncated F2 domain identified its consensus N-terminal amino acid sequence (Met-Glu-Glu) and revealed a proteolytic cleavage site within EBA-140 F2 domain (Fig. 6). It allows to identify and calculate the mass of the 213 amino acid protein consisting of 197 aa residues of the F2 domain and tags (16 aa). The molecular mass of the truncated F2 domain, evaluated by mass spectrometry was 26 300 Da and it corresponded exactly to the molecular mass calculated according to the amino acid sequence.

Conformation analysis of truncated F2 domain

Proper folding of the recombinant EBA-140 truncated F2 domain was confirmed by circular dichroism. The CD spectrum of the purified, truncated F2 domain showed minima near 208 and 220 nm, similarly to the recombinant Region II of the EBA-140 ligand obtained in the baculoviral system (Fig. 7). These results indicate the presence of a significant α-helical content in the EBA-140 truncated F2 domain.

Functional analysis of EBA-140 truncated F2 domain

The ability of the truncated F2 domain to bind its ligand was evaluated by examining its interaction with native and neuraminidase or trypsin treated RBC in flow cytometry (Fig. 8). Binding of the truncated F2 domain was compared to that of baculovirus-expressed Region II of EBA-140 antigen. It was shown that the truncated F2 domain could bind to native human erythrocytes, but did not bind to neuraminidase- or trypsin-treated RBC, similarly to the whole recombinant Region II. These results indicated that the recombinant, truncated F2 domain, although comprising only 197 of the 335 amino acid residues of the F2 domain, was still functional and could bind specifically to human erythrocytes in a sialic acid dependent manner. However, binding of the truncated F2 domain to RBC evaluated by flow cytometry was much weaker than binding of the full-length Region II of EBA-140 antigen, even at high concentration.

It was shown that the recombinant Region II of *P. falciparum* EBA-140 antigen expressed on the surface of CHO-K1 cells bound to native but not to neuraminidase-treated erythrocytes (Jiang *et al.*, 2009). Similar results were obtained previously for the whole EBA-140 Region II expressed on the surface of COS7 cells (Mayer *et al.*, 2002). Moreover, it was also observed that the F1 and F2 domains of EBA-140 Region II expressed separately on the surface of COS7 cells did not bind to human erythrocytes, which may suggest that both domains are simultaneously required for binding (Mayer *et al.*, 2002). This observation was recently confirmed and demonstrated for F1

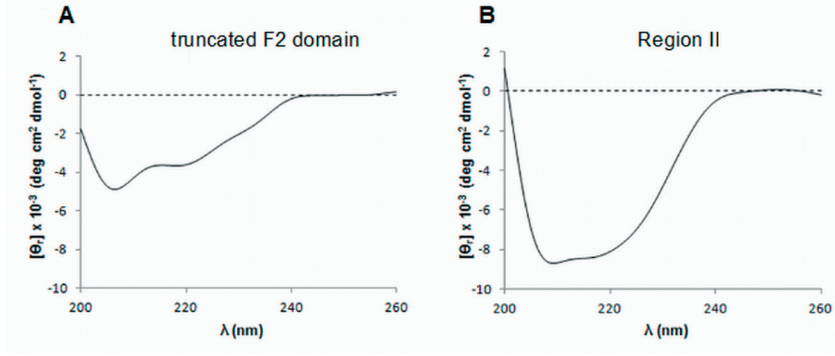

Figure 7. Circular dichroism spectra (195–270 nm) of EBA-140 truncated F2 domain (A) and whole Region II (B);
solutions of the truncated F2 domain (2 μM) and Region II (0.5 μM) in 50 mM Tris/HCl, 300 mM NaCl, 10% glycerin, pH 7.5 were used.

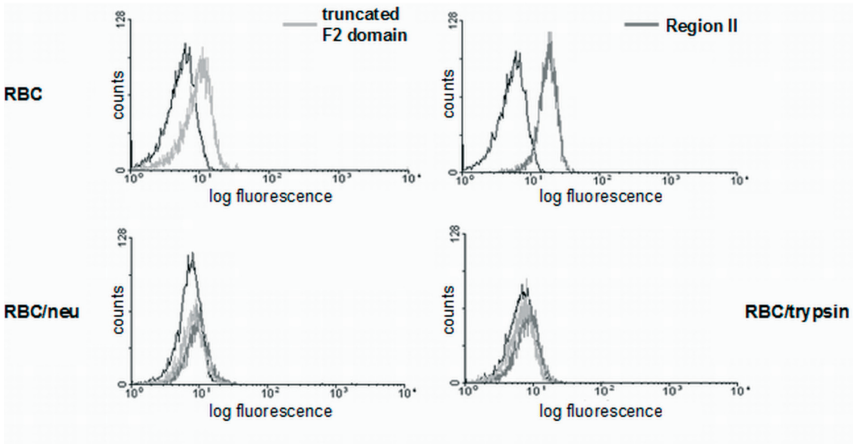

Figure 8. Flow cytometry analysis of the truncated F2 domain and full length Region II binding to native (RBC) and neuraminidase (RBC/neu) or trypsin (RBC/trypsin) treated human erythrocytes.
Black line corresponds to control (erythrocytes incubated with anti-Region II rabbit serum); light grey line corresponds to the truncated F2 domain; dark grey line corresponds to Region II of EBA-140 antigen.

and F2 domains expressed individually on HEK-293T cells and tested for erythrocyte binding (Lin *et al*., 2012). In contradiction to EBA-140 antigen, it was shown that the recombinant Region II and F2 domain (but not F1 domain) of EBA-175 homologous antigen bound human erythrocytes in a similar manner (Liang *et al.,* 2000; Ockenhouse *et al.,* 2001; Pandley *et al*., 2002). These data suggest that the most important region required by EBA-175 antigen to bind to its GPA ligand is the F2 domain.

Our findings have demonstrated that the truncated F2 domain of EBA-140 antigen can still specifically bind to erythrocytes, although its binding is not as efficient as binding of the whole Region II. This confirms that both F1 and F2 domains of EBA-140 antigen are required for effective erythrocyte binding as shown previously (Mayer *et al*., 2002; Lin *et al*., 2012). However, in comparison with the total loss of activity shown for the EBA-140 F2 domain expressed as surface recombinant protein and examined for erythrocyte binding by rosetting assay, the soluble, recombinant and truncated F2 domain can still bind to erythrocytes, as confirmed by flow cytometry. The observed discrepancies between the results can be explained by the sensitivity of methods employed.

CONCLUSIONS

The EBA-140 ligand is a member of the *P. falciparum* EBL family of proteins responsible for human erythrocyte binding. This paper describes bacterial expression and purification of the recombinant, truncated F2 domain of the *P. falciparum* EBA-140 ligand. Two major conclusions can be drawn from this study.

The first conclusion is that the soluble, pure and properly folded truncated F2 domain (197 aa res.) of EBA-140 antigen could be obtained in milligram quantities in bacteria.

The second and most important conclusion is that the truncated F2 domain obtained by this method is functional and can bind to human erythrocytes, although its binding is not as efficient as that of the full-length Region II (F1 and F2 domains) of EBA-140 antigen. It is consistent with the notion that both F1 and F2 domains of EBA-140 antigen are required for effective erythrocyte binding.

Acknowledgemets

The research was supported by grant No N N302 281436 from the Ministry of Science and Higher Education of Poland.

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