NEW BIOMEDICAL TECHNOLOGIES

Expression of Human IgE-Binding Factor (sCD23) in *Escherichia coli*

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Previously, we cloned cDNA coding for type II Fc receptor for human IgE (CD23 or FcERII). A fragment of this cDNA coding for soluble IgE-binding factor fused with a fragment of human interleukin-3 gene was cloned in pBT-IL-3-sCD23 plasmid. It is demonstrated that *E. coli* strain JM109 (pBT-IL-3-sCD23) expresses the hybrid protein with a yield of 10-15% total cell protein. The recombinant product is represented by equal amounts of two polypeptides with molecular weights of about 24 and 32 kD. Immunological analysis and determination of the amino acid sequence of the N-terminal ends of these polypeptides show that a protein with a molecular weight of 24 kD results from proteolysis of the full-size (32 kD) hybrid protein. The preparation obtained can be used for the development of test kits for CD23.

Key Words: *low-affinity Fc receptor of human IgE (CD23); expression in E. coli; hybrid protein IL-3-sCD23*

The high prevalence of allergic disorders prompts investigation into the regulatory mechanisms of the biosynthesis of immunoglobulin E (IgE), a key component in hypersensitivity reactions of the immediate type. A low-affinity fragment of the receptor for the Fc fragment of human IgE (FcERII), which was identified as B-cell differentiating antigen CD23 [5], is involved in the regulation of IgE biosynthesis [5]. FcERII (CD23) is a single-chain transmembrane type II glycoprotein with a molecular weight of 45 kD containing one complex N-glucoside chain and several O-glycoside chains with sialic acid residues [14]. This receptor is present on eosinophils, monocytes, platelets, and B and T cells. It participates in IgE-dependent cytotoxic reactions to parasite invasions and IgE-dependent antigen presentation as well as in the regulation of IgE biosynthesis and B-cell growth and differentiation [6]. Membrane-bound receptors are cleaved autoproteolytically to form a number of soluble (secreted) fragments with molecular weights of 37, 33, 29, 25, and 16 kD, which have been termed IgE-binding factors (IgE-BF or sCD23) [11]. These factors are involved not only in IgE binding *in vivo*, but also in a number of other biological reactions *in vitro*. For example, sCD23 stimulates the growth of some blood cell populations, regulates IgE synthesis *in vitro*, and the proliferation of T cells, inhibits the migration of monocytes, and, together with interleukin-1 (IL-1), promotes the differentiation of young thymocytes and the survival of B cells in the germinal centers of lymph nodes [6]. These functions may be mediated by the recently discovered interactions between CD23 and CD21 [4]. Cloning of cDNA coding for human CD23 has been performed in several labo-

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ratories [8,10,12], and the structure and function of recombinant proteins have been studied. However, a system for the expression of recombinant CD23 in prokaryotic cells has not been developed. There has been only one successful attempt to achieve highly efficient expression of the full-size protein and its fragments in eukaryotic cells (Sf9) with the use of a baculoviral system [9]. Although the expression of recombinant polypeptides was quite high, another pattern of protein glycosylation may alter the biological activity of the resulting preparations. In the present study we constructed an expressing plasmid that yielded a high (10-15% of total cell protein) production of the recombinant hybrid protein sCD23 by *E. coli* and developed a method for isolating and purifying this protein. It is shown that the preparation reacts with polyclonal antibodies against a synthesized fragment of sCD23.

MATERIALS AND METHODS

Plasmid p121E1 containing full-size cDNA coding for CD23 was obtained as described previously [2]. To construct the expressing vector we used plasmid pBT-IL-3-10, which has been reported to provide effective synthesis of IL-10 [3]. The CD23 gene without nontranslated terminal regions was obtained by polymerase chain reaction on plasmid p121E1 (direct primer 5'-CCGCTCGAGATGGAGGAAGGTCAATATTCAGAG, reverse primer 5'-CCGCTCGAGTCAAGAGTGGAGAGGGGCAGAGGG, cycle parameters: 94°C — 1 min, 56°C — 1 min, 72°C — 1 min) and subcloned in plasmid pUC18 at the Sma I site. The Hind III—Pst I fragment of this construction contains a fragment of the CD23 gene coding for the C-terminal sequence of the molecule from Arg-133 and a fragment of polylinker with the sites for Xho I, BamH I, Xba I, Sal I, and Pst I restriction endonucleases. This fragment was isolated by preparative electrophoresis in agarose gel and cloned in pBT-IL-3-10-Hind III—Pst I vector. A schematic drawing of pBT-IL-3-sCD23 plasmid is given in Fig. 1. Standard techniques were employed for all genetic engineering manipulations [13]. The producer *E. coli* strain JM109 (pBT-IL-3-sCD23) was grown in Luria-Broth Base, Miller (LB) medium with ampicillin (100 μg/ml) at 37°C with intense aeration to optical density A_{500}=1. Protein production was induced by the addition of 0.5 mM isopropylthiogalactoside (IPTG) and was monitored at different periods of incubation. Protein preparations were analyzed by 12.5% polyacrylamide gel electrophoresis (PAGE) (bis:acrylamide ratio 1:25) in the presence of sodium dodecyl sulfate (SDS). Recombinant protein was isolated and purified as follows:

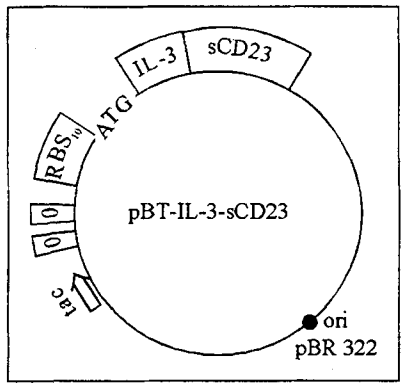

Fig. 1. Diagram of plasmid pBT−IL−3−sCD23. *tac* is the promoter; *0* is the operator binding site; RBS_{10} is the binding site for the ribosome of bacteriophage T7 protein 10; *ATG* is the start codon; *IL−3* is a fragment (177 bp) of the IL−3 gene. The solid line shows the pKK 223−3 plasmid (Pharmacia).

0.1 g wet cells was resuspended in 1 ml lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 100 mM NaCl), and phenylmethylsulfonylfluoride and lysozyme were added to final concentrations of 1 mM and 1 mg/ml, respectively. Incubation was carried out on ice with constant stirring for 20 min. Dry deoxycholic acid (1 mg) was then added and stirred at 37°C until the lysate condensed, after which 200 μg DNAase I were added, and the lysate was left at room temperature until it lost its viscosity. The lysate was centrifuged (10,000 g, 10 min), and the pellet was washed with nine volumes of lysis buffer containing Triton X-100 and 10 mM EDTA. The preparation was purified by successive washings in 1 ml of 1, 2, and 3 M guanidine-HCl with 0.5% Triton X-100. The protein concentration in the preparation was measured by the micromethod of Lowry. Part of the pellet containing 1 mg protein was dissolved for 1 h at 37°C in 400 μl buffer (0.1 M Tris-HCl, pH 9.0, 6 M guanidine-HCl, and 0.2 M dithiothreitol), and undissolved protein was removed by centrifugation (10,000 g, 30 min). The solution was slowly (1 h) diluted in buffer (1 M guanidine-HCl, 20 mM Tris-HCl, pH 9.0, 0.25 M NaCl, and 0.01% Nonidet P-40, a nonionic detergent) to a final volume of 8 ml. The precipitate was removed by centrifugation. The preparation was incubated for another 2 h at room temperature and dialyzed against 3 volumes of buffer containing 0.5 M guanidine-HCl and 20 mM Tris-HCl (pH 9.0) overnight at 4°C. It was then dialyzed for 4 h at 4°C against 6 volumes of buffer (0.15 NaCl, 0.3 M guanine-HCl, 20 mM Tris-HCl, pH 9.0) and further dialyzed against two liters of buffer containing 150 mM NaCl and 10 mM Tris-HCl, pH 8.0) at 4°C overnight without stirring. The protein solution was concentrated to 0.3 mg/ml in a dialysis bag with the use of dry polyethyleneglycol (20,000, Loba). The

precipitate was removed by centrifugation (10,000 g, 30 min). The preparation was stored in aliquots at -20°C. Antiserum was obtained by immunizing a rabbit with bovine serum albumin (BSA) conjugated to chemically synthesized peptide corresponding to the Gly-178/Glu-195 fragment of CD23. The conjugate was obtained with the use of glutaraldehyde [7]. Specific antibodies were isolated by affinity chromatography on the peptide conjugated with ovalbumin.

For immunoblotting the preparation was electrophoresed in 12.5% SDS PAGE with and transferred onto a nitrocellulose membrane by the semidry method [7]. To reduce nonspecific binding the blot was incubated in standard saline buffer with 3% BSA and 20% horse serum for 16 h at 4°C. Incubation with antibodies was carried out in the same buffer. Phosphate-buffered saline with 0.05% Tween-20 was used for washing. Specific antibodies were diluted to a final concentration of 2 μg/ml; control serum was used in a 1:100 dilution. Specifically bound antibodies were detected with the use of goat antibodies against rabbit IgG conjugated with horseradish peroxidase (BioRad) (dilution 1:2000). The substrate buffer contained 50 mM Tris-HCl, pH 7.5, 0.003% cobalt chloride, 0.2 mg/ml imidazole (Merck), 0.2 mg/ml 3,3-diaminobenzidine (Sigma), and 0.03% hydrogen peroxide. The amino acid sequence was determined as described elsewhere [1].

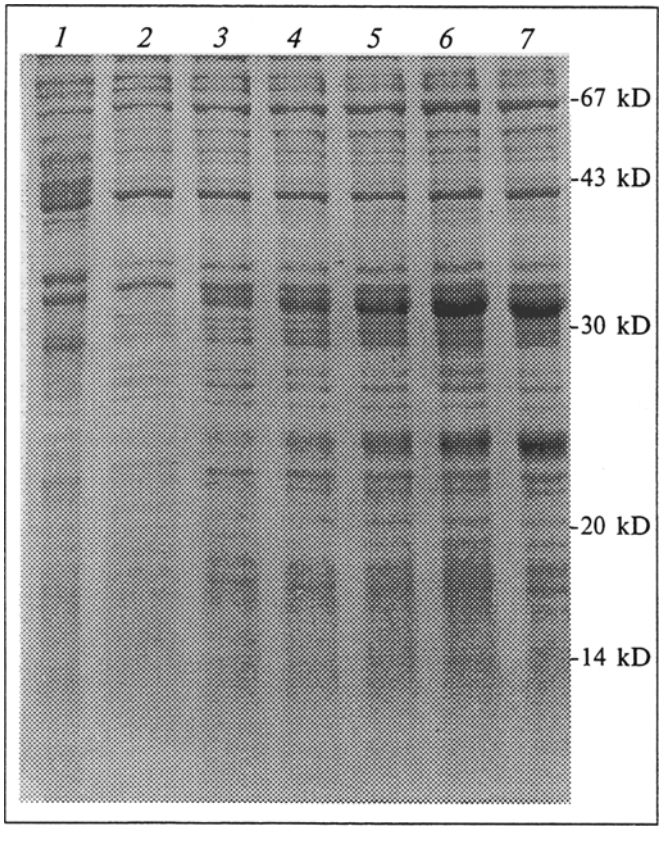

Fig. 2. Electrophoregram of the total cell protein of *E. coli* strain JM109 (pBT−IL−3−sCD23). *1*) JM109 strain without plasmid (control); *2*) JM109 (pBT−IL−3−sCD23) grown without the inducer isopropylthiogalactoside (IPTG); *3*) 30 min after the addition of IPTG; *4*) after 1 h; *5*) after 2 h; *6*) after 3 h; *7*) after 4 h.

RESULTS

In order to obtain a recombinant preparation of CD23 we used plasmid in which cDNA coding for the full amino acid sequence of CD23 was inserted. At a distance of 50 base pairs (bp) from the beginning of 25 kD IgE-BF this cDNA contains a unique Hind III site which was used to obtain a fragment of the gene coding for soluble CD23. This fragment was cloned into the expressing plasmid pBT-IL-3-10. It was previously demonstrated that this construction provides a very high level of IL-3-10 expression [3]. Cloning of the CD23 fragment at the Hind III site allowed us to fuse the CD23 and IL-3 sequences and to preserve the reading frame without any additional manipulations. The inserted fragment encodes the C-terminal region of CD23 starting from Glu-133 and includes the 25 kD IgE-BF that starts from Arg-149. Thus, the construction obtained codes for the hybrid polypeptide IL-3-sCD23 consisting of 62 amino acid residues of the N-terminal domain of IL-3 and 188 residues of the C-terminal domain of CD23.

It should be noted that we failed to develop a system for direct expression of mature sCD23 (25 kD). The gene coding for sCD23 was cloned into plasmid pBT-IL-3 under the control of the *tac* promoter and the binding site for the ribosomes of bacteriophage T7 protein 10 and into plasmid pBT-hEGF as a gene fused with the gene coding for the leading sequence of OmpF. The first and second variants should have provided for the synthesis of the protein in the cytoplasm and in the periplasm of *E. coli*, respectively. However, in neither case did we detect recombinant protein (data not shown). Presumably, in the cytoplasm sCD23 underwent intensive proteolysis and had a toxic effect after being secreted into the periplasm, which led to lysis of *E. coli* cells after the induction of protein biosynthesis.

Plasmid pBT-IL-3-sCD23 was used to transform cells of *E. coli* strain JM109, and the expression of recombinant protein was studied under conditions of repression and derepression of the *tac* promoter. The cells were grown in LB medium until A_{500}=1 was reached, protein synthesis was induced by the addition of IPTG, and samples were collected after 30 min, and 1, 2, 3, and 4 hours. The level of recombinant protein production was assessed by SDS PAGE. As shown in Fig. 2, two products with molecular weights of 32 and 24 kD appear during

the incubation. The larger polypeptide is formed predominantly in the early stages, but by the 4th hour both are produced in an almost equal ratio, amounting to 20-25% of the total protein content. Recombinant protein was identified with the use of polyclonal rabbit antibodies against chemically synthesized peptide from the N-terminal region of 25 kD IgE-BF. This peptide was selected with the use of computer software devised by Dr. P. Zhilkin (Vektor, Russia) for predicting the antigenic determinants of a protein molecule. The antipeptide antiserum was used because there are no commercial antibodies reacting with denatured CD23 with the required specificity. The results of immunoblotting are shown in Fig. 3, *a*. In crude lysate the antipeptide antibodies reacted only with the recombinant protein (track *2*), the interaction being equally strong with both protein bands, while nonimmune rabbit antiserum (track *3*) reacted with a number of bacterial proteins but not with the recombinant product. The same *E. coli* strain carrying an analogous plasmid in which the IL-10 gene was inserted instead of CD23 was used as the control. Amino acid analysis of the N-terminal region showed the same sequence in both products: N-Met-Ala-Pro-Met-Thr-Gln, which coincides with the amino acid sequence of IL-3. Interestingly, the molecular weight of the hybrid protein, calculated as the sum of amino acid residues, is 28 kD, whereas on the electrophoregram the recombinant product is represented by two bands with apparent molecular weights of around 32 and 24 kD. Based on the assumed abnormal electrophoretic mobility of the protein, which is quite possible due to, for example, an increased content of proline (8% *vs.* an average value of 4%), we believe that the upper band (32 kD) corresponds to the full-size hybrid protein, while the lower band (24 kD) probably represents a product of proteolysis; the site at which the molecule is cleaved is probably located in the C-terminal region of the molecule.

Assuming the hybrid protein to form inclusion bodies, we purified the preparation as follows: cells were lysed by lysozyme in the presence of deoxycholic acid and centrifuged. Analysis of the supernatant and pellet showed all recombinant protein to be in an insoluble form. The protein was purified by successively washing the pellet with ascending concentrations of guanidine-HCl (from 1 to 3 M). The product loss was <20%, and the content of protein contaminants dropped to 10%, as evidenced by electrophoresis. The inclusion bodies were solubilized by dissolving the pellet in 6 M guanidine-HCl with 0.1 M Tris-HCl, pH 9.0, and 0.2 M dithiothreitol at 37°C. It was assumed that under these conditions the protein would be completely denatured. For its renaturing the solution was slowly (during 1 h) diluted with a buffer containing 1 M guanidine-HCl to a final guanidine-HCl concentration of 1.33 M. The preparation was then dialyzed overnight against 3 volumes of buffer with 0.5 M guanidine-HCl, the guanidine-HCl concentration being slowly lowered to 0.7 M. Attempts to speed up renaturing by dilution or dialysis against lower concentrations of guanidine-HCl led to precipitation of 90% recombinant product. The preparation was then dialyzed for 4 h against 6 volumes of 0.3 M guanidine-HCl and overnight against 250 volumes of 150 mM NaCl with 10 mM Tris-HCl, pH 8.0. During renaturing, the preparation was further purified; however, 25% of the protein coprecipitated with contaminants. In the last stage of purification, the preparation contained >90% recombinant product. Electrophoresis and immunoblotting showed the preparation to contain almost equal amounts of the two polypeptides with molecular weights of about 24 and 32 kD (Fig. 3, *c*, track *1*). Both polypeptides react with specific rabbit antibodies against the peptide fragment of sCD23 (track *2*) but not with nonimmune rabbit antiserum (track *3*).

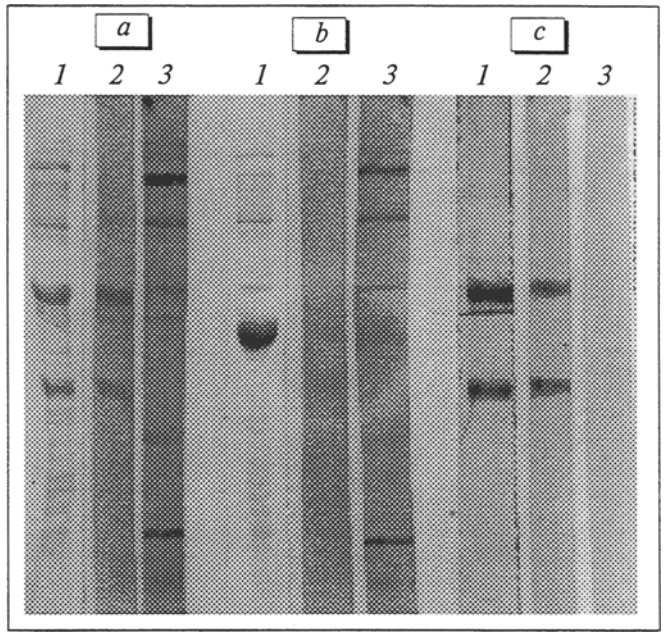

Fig. 3. Immunoblotting. *a*) lysate of *E. coli* JM109 (pBT−IL−3−sCD23); *b*) lysate of the same cells taken as the control for antibody specificity: on the electrophoregram the hybrid protein IL−3−10 is clearly seen in the 28 kD region; *c*) purified recombinant preparation. *1*) electrophoregram; *2*) blot stained with antibodies against peptide fragment CD23; *3*) blot stained with nonimmune rabbit serum.

Thus, a procedure was developed for the purification and solubilization of recombinant product from inclusion bodies. At present, we are working on separating the polypeptides and determining their

C-terminal amino acid sequences to localize the proteolysis sites.

A modification of the construction by creating a site for a highly specific protease in the hybrid protein is planned, which will allow us to obtain an sCD23 preparation without additional sequences and to develop a method of restoring the native structure of the protein. This preparation can be used for the production of monoclonal antibodies and the development of test kits. In conclusion it should be noted that different CD23 variants are necessary for the investigation of the regulatory mechanisms of IgE biosynthesis and for the construction of IgE analogs with a higher physiological activity, which may find clinical application in the treatment of allergic disorders.

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