GENETICS

Production and Characterization of a Recombinant Ligand-Binding Domain of the Human Low-Density Lipoprotein Receptor

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Two plasmids carrying the cDNA fragment of human low-density lipoprotein receptor coding for its ligand-binding domain are constructed. The ligand-binding domain of the receptor composed of fused and individual polypeptides is purified and characterized.

Key Words: *recombinant proteins; lipoproteins; membrane receptors*

Various mutations in the gene coding for the low-density lipoprotein (LDL) receptor lead to an autosomal dominant disease, familial hypercholesterolemia [4,7]. The biochemical phenotype of this disease may manifest itself in a quantitative deficiency of LDL receptors on the cell surface or in the synthesis of a receptor protein lacking functional (LDL-binding) activity. Functional analysis of mutant alleles of the LDL receptor gene has been performed with the use of a test system consisting of receptor-deficient Chinese hamster cells (CHO-ldlA line) transfected with recombinant constructs [10]. The system is not very convenient for such an analysis, therefore, it is desirable to develop an alternative cell-free system for evaluating the function (ligand-binding activity) of mutant LDL receptor forms. The present study represents an attempt to produce a ligand-binding domain of human LDL receptor in the form of a recombinant protein. To this end, the cDNA fragment of the LDL receptor encoding its ligand-binding domain was cloned into two bacterial plasmids, followed by physicochemical and immunological analysis of their expression products which are recombinant proteins.

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MATERIALS AND METHODS

The material was the full-length cDNA of the human LDL receptor inserted into pLDLR3 plasmid [14]. The Pst I fragment of the plasmid DNA 1100 base pairs (bp) long was isolated by electrophoresis in a low-melting agarose [2] and cloned for the unique Pst I site of pUEX2 plasmid [3,13]. This construction was used to transform *E. coli* cells of the DH5α strain. Recombinant colonies were selected using hybridization of the original 1100 bp Pst I fragment of pLDLR3 ^{32}P, which was labeled using DNA polymerase I, and statistical primers. The plasmid DNA was isolated by alkaline lysis [2], and the orientation of the insert relative to the plasmid promoter was determined with the restriction endonuclease EcoR I which cleaved the recombinant DNA at asymmetric site within the insert and at a second site in the vector polylinker. DH5α cells transformed by the control vector plasmid or by the selected chimeric plasmid pUEXL45 were grown at 32°C, and expression of the cloned gene was induced by elevating temperature to 43°C, which inactivated the thermo-

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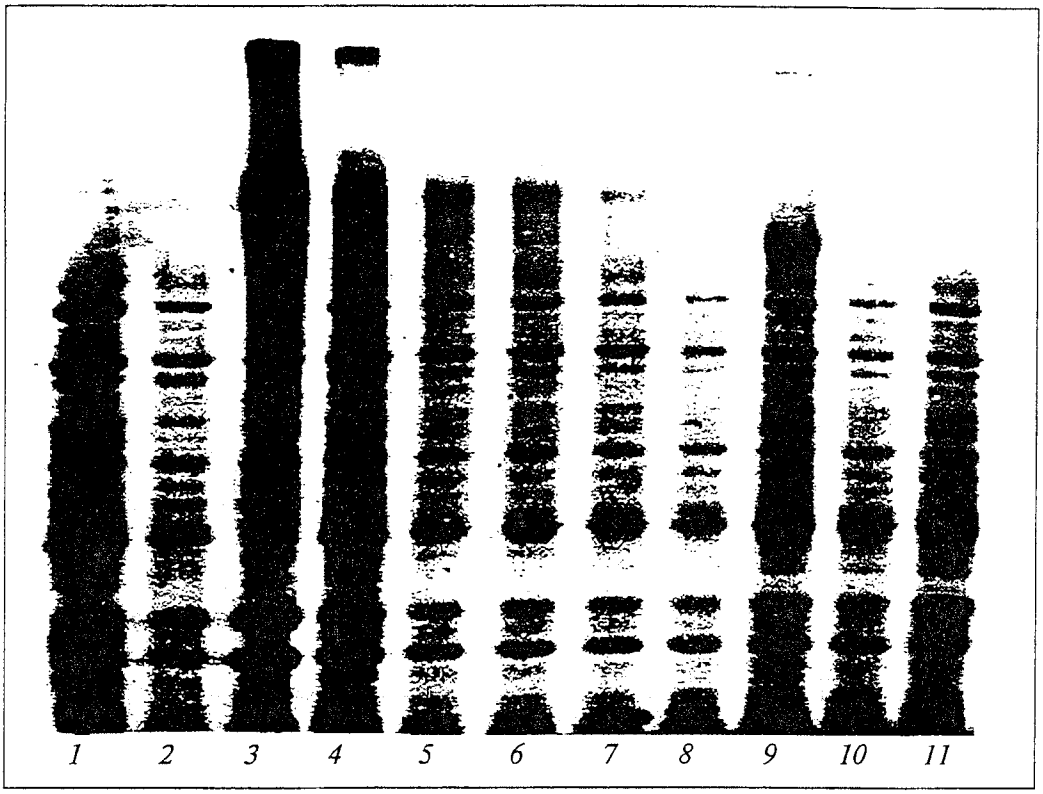

Fig. 1. Optimization of conditions for inducing synthesis of a fusion protein containing the ligand-binding domain of the LDL receptor, and partial purification of the protein. *1* and *2*) supernatants obtained by sequential washing of inclusion bodies from pUEXL45 plasmid-transformed DH5α cells; *3*) preparation of inclusion bodies; *4*) ultrasonic culture lysate; *5-11*) ultrasonic lysates of cultures of the plasmid-free DH5α strain: after 60 min of induction (*11*), with the plasmid vector pUEX2 after 60 min of induction (*9*) and without induction (*10*), and with the plasmid vector pUEXL45 without induction (*8*) and after 90 (*5*), 60 (*6*), and 30 (*7*) min of induction. Here and in Fig. 2: denaturing electrophoresis was performed in 7.5% polyacrylamide gel and the proteins were stained with Coomassie R-250.

labile C1 repressor of phage λ regulating transcription from the λP_L promoter in the plasmid vector. After determining the optimal time for induction, partial purification of the recombinant protein was carried out [12] (Fig. 1). A recombinant protein isolated directly from the appropriate electrophoretic zone was used to immunize rabbits.

For cloning in the plasmid vector pET22b(+), the pLDLR3 plasmid DNA fragment encoding the ligand-binding domain of the LDL receptor was amplified using two primers; the left primer was inserted into the Hind III site and the right primer into the Xho I site during synthesis. The amplification product and pET22b(+) vector were digested with Hind III and Xho I and then ligated at the equimolar vector/amplification product ratio. Ligase mixture was used to transform *E. coli* DH5α cells, and the plasmids isolated from the transformed colonies of these cells were transferred to the expressing strains BL21(DE3) and BL21(DE3)pLysS (Fig. 2). Subcultures were grown to the late logarithmic phase (A_{600}=0.6), after which isopropyl thiogalactoside (IPTG) was added to a concentration of 1 mM to induce synthesis of T7-RNA polymerase. Cells were collected 3 h later by centrifugation and sonicated. Proteins of the periplasmic space were obtained by chloroform treatment. Secretory proteins were precipitated from the culture medium with ammonium sulfate at 80% saturation and dialyzed. The recombinant ligand-binding domain with its C-end tail $(His)_6$ was isolated in an affinity column containing a nickel ion-chelating resin according to the manufacturer's instructions. The chimeric protein was identified by immunoblotting using our antibodies after SDS-7.5% polyacrylamide gel electrophoresis and semidry transfer to Hybond C-extra nitrocellulose membranes (Amersham). Incubation of the membrane was carried out with detection of the bound immunoglobulins using staphylococcal G protein or peroxidase-conjugated antibodies to rabbit immunoglobulins [12]. The chromogenic substrate for the peroxidase was 4-chloro-1-naphthol. Biological activity of the recombinant ligand-binding domain of the LDL receptor was determined by ligand blotting [5].

RESULTS

The fragment of the LDL receptor full-length cDNA coding for the ligand-binding domain of this receptor

and the cysteine-rich A repeat of the second domain, i.e., the receptor domain minimally required for ligand-binding activity according to deletion analysis [8], was recloned into two plasmids. The first chimeric construct (pUEXL45) enabled us to obtain a fused protein in which the sequence of interest was located at the C-end of the polypeptide which also contained sequences of phage λ *cro* protein and of bacterial β-galactosidase. This fusion protein, which has a higher molecular weight than the host cell proteins and the β-galactosidase encoded by the control vector plasmid pUEX2, was readily detectable upon electrophoresis of lysates from the induced cultures (Fig. 1). The recombinant protein formed in the cytoplasm insoluble inclusion bodies which could be viewed during light microscopy of cells from the induced cultures. Separation of the inclusion bodies resulted in partial purification of this protein. For the immunization of rabbits, the electrophoretic zone whose molecular weight corresponded to that of the recombinant fusion protein was used. The immunization gave rise to an antiserum that retained, after its depletion with *E. coli* DH5α pUEX2 cell proteins [12], the capacity for interaction with the material used for the immunization, but did not react with other *E. coli* proteins. This antiserum was used for immunological identification of the recombinant ligand-binding LDL-receptor protein synthesized in another system — BL21(DE3)pETL with the IPTG-induced RNA polymerase of phage T7. The chimeric protein made up less than 5% of the total cellular protein rather than 15-20% as in the first system, but was synthesized as an individual (nonfused) polypeptide containing only an excess C-end tail $(His)_6$, which was used for affinity purification of this protein, and a leader sequence for its secretion into the periplasmic space and into the culture medium. This recombinant protein with antigenic specificity of the LDL receptor ligand-binding domain was detected by immunoblotting in unfractionated cell lysates, in the periplasmic space, and, in large amounts, in the culture fluid. The protein was isolated by affinity chromatography on immobilized nickel as a highly (>80%) purified preparation and used for rabbit immunization.

The antibodies to the two (fusion and nonfusion) proteins containing sequences of the LDL-receptor ligand-binding domain reacted positively upon immunoblotting with proteins of cultured human fibrosarcoma cells. It is therefore likely that these antibodies will find application for diagnosing those forms of familial hypercholesterolemia which involve a quantitative deficiency of LDL receptors on the surface of cells such as fibroblasts and lymphocytes. As a negative control in such tests, cells from a female patient with deleted LDL-receptor ligand-binding domain [1] can be used.

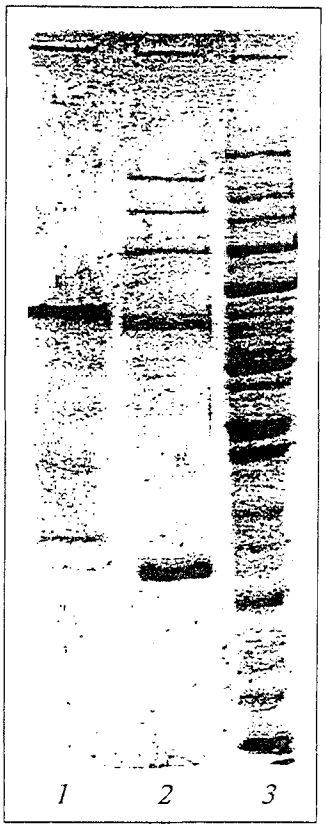

Fig. 2. Purification of the recombinant ligand-binding domain of the LDL receptor by affinity chromatography. The soluble fraction of ultrasonic lysates of induced BL21(DE3)pLysS pETL culture were applied to a column chelating Ni^{2+} ions and then eluted in sequence with three buffer solutions in a discontinuous imidazole concentration gradient (lines *3*, *2*, and *1*, respectively).

The native recombinant protein with antigenic properties of the LDL-receptor ligand-binding domain exhibited in our preliminary experiments a very low LDL-binding activity which became increasingly lower as this protein was purified. Further experiments should disclose the reasons for its inadequate functional activity. Possible reasons include absence in the bacterial cells of N— and O-glycosylation reactions, which play a role in the regulation of ligand binding to the LDL receptor [6,9], and improper formation of disulfide bonds between cysteine residues in the recombinant protein. The latter possibility appears more plausible since breakage of disulfide bonds in the extracellular domain of the LDL receptor has been shown to disrupt completely its binding to a LDL ligand [5], and since the proper formation of disulfide bonds probably occurs specifically upon cotranslational translocation of proteins into the lumens of endoplasmic reticulum cisternae [11]. Functionally active recombinant ligand-binding domain of the LDL receptor can probably be obtained after obviating these complexities through the use of the protein disulfide isomerase (an enzyme which accelerates the formation of proper disulfide bonds), eukaryotic chaperons, or yeast system.

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