EXPRESSION OF LBP 32/67 kD HUMAN GENE IN *E. coli* AND ANALYSIS OF ITS BINDING WITH LAMININ

E. Yu. Siyanova

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Processes involved in the onset and development of tumors are intimately connected with the state of the extracellular matrix, with the ability of cells to respond to the matrix, and with the work of the receptor apparatus of the cells [4]. Previously the writer described cloning and determination of the primary sequence of the gene of human laminin-binding protein (LBP) [1]. The molecular weight of the predicted protein is 32 kD. This gene also has been cloned in Liotta's laboratory [6] with the aid of antibodies to a protein with molecular weight of 67 kD. The question arises, how these proteins are linked with each other and whether the protein with mol. wt. 32 kD can bind with laminin. To test this possibility, it was decided to obtain expression of the LBP 32 kD gene in *E. coli* cells and to carry out function tests on the hybrid protein thus obtained.

To analyze binding of the LBP-TrpE component protein with laminin we used three different approaches: immunoblotting, ELISA, and affinity chromatography. By considering all the data obtained it was possible to conclude that this protein binds specifically with laminin.

EXPERIMENTAL METHOD

As the vector of expression we used plasmid vector pATH-LBP, containing the TrpE-promoter and sequence for 37 kD TrpE protein, located before the mp13 multicloning site for insertion of the required sequence [5].

To obtain the LBP-$TrpE_1$ protein we used strain CAG-456 [5], containing plasmid pATH-LBP. An overnight culture of this strain was diluted with 4 volumes of medium M9-CA, containing 50 μg/ml ampicillin, and incubated at 30°C. Expression of the component protein was induced by the addition of IAA in a final concentration of 10 . After incubation for 5 h at 30°C, PMSF (protease inhibitor) was added to a final concentration of 0.2 mM. The cells were sedimented by centrifugation and the residue suspended. The residue was resuspended in lytic buffer (3 ml buffer per gram of residue). The sample was incubated at 37°C for 10-20 min, DNase I was added (20 μl per gram of residue), and the sample was incubated at room temperature until its viscosity disappeared. After centrifugation (12,000*g*, 4°C, 15 min) the supernatant was removed and the residue resuspended in 9 volumes of lytic buffer (0.5% Triton X-100, 10 mM EDTA, pH 8.0). After incubation for 5 min, the sample was centrifuged at 12,000*g*, 4°C, for 15 min, the supernatant was removed, and the residue, consisting of LBP-TrpE component protein, was resuspended in lytic buffer with 8 M urea and PMSF.

For ELISA the plates were covered with component protein (100 μl per well) and incubated for 16 h at 4°C. The protein concentration was 5-10 μg/ml in 0.1 M bicarbonate buffer, pH 9.5. After triple washing in PBS/Tween buffer the plates were incubated with laminin (10 μg/ml – 10 pg/ml) for 2-4 h at 37°C. Antibodies (AB) to laminin were then added and the sample incubated for 1 h at 37°C. Rabbit antimouse AB with alkaline phosphatase in a dilution of 1:1000 were then added. After washing the plates were incubated with the substrate.

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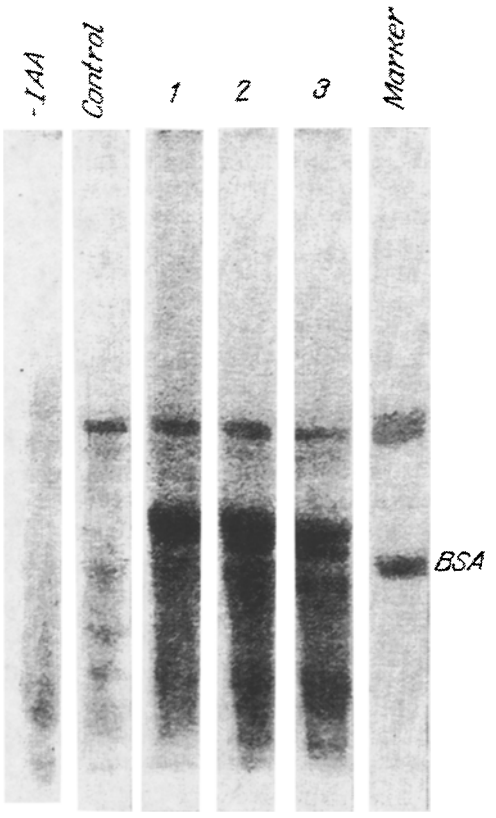

Fig. 1. Analysis of expression of LBP-TrpE component protein in *E. coli* cells. PAG stained with Coomassie. Cell lysates applied to lanes of the gel: IAA) Lysate from cells grown without inducer; control) lysate from cells transformed by plasmid without LBP gene; 1, 2, 3) lysates from cells of different clones, containing vector with LBP gene; marker) marker proteins. Arrow on left indicates location of hybrid protein.

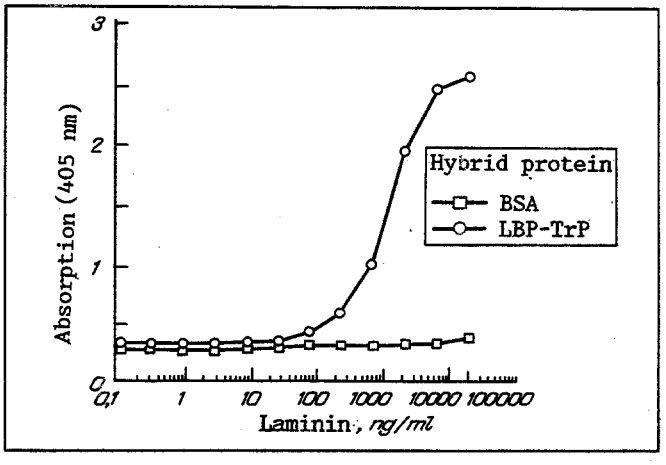

Fig. 2. Analysis of binding of laminin with LBP-TrpE protein by ELISA. "Microtiter" plates covered with hybrid protein TrpE-LBP or BSA. Intensity of staining estimated on spectrophotometer.

For affinity chromatography the ^{35}S-component protein was incubated with laminin-sepharose and gelatin-sepharose at room temperature for 2-4 h. The resin was then washed 3 times with TBS, 3 times with 1 M NaCl, and 3 times with 8 M urea. The quantity of label at each stage was determined. An aliquot from each washing was applied to SDS-polyacrylamide gel (SDS-PAG). The dried gel was subjected to autoradiography.

EXPERIMENTAL RESULTS

The test gene may be cloned in one reading frame with synthetic or bacterial coding sequences and expressed with the formation of a hybrid protein. The need for eukaryotic polypeptides in the composition of the hybrid proteins arose in expression when it was found that the level of expression of eukaryotic proteins in *E. coli* cells is limited, the reason being that they are recognized by the cell as foreign and are destroyed [2]. During splicing of the eukaryotic gene with the bacterial gene, hybrid products are synthesized and these accumulate in the cell in considerable amounts. To obtain the eukaryotic polypeptide in a pure form, accurate cleavage of the hybrid protein is essential. For this purpose a hybrid gene is constructed which has a cleavage point in its product located between the bacterial and eukaryotic gene, for example, a tetrapeptide sequence recognized by the Xa blood clotting factor. To clone the test sequence, special oligonucleotides were synthesized. Oligonucleotide 1 (41 bp) contains, starting from the 5′-end, an EcoRI site, for cleavage by the Xa blood clotting factor, initiating the codon of the gene of laminin-binding protein, and a sequence corresponding to the first 6 N-terminal amino-acids of this protein:

5′—TA—ACG—AAT—TCG—ATA—GAA—GGC—
AGA—ATG—TCC—GGA—GCC—CTT—GAT—3′
[1].

The second oligonucleotide (40 bp) contains a site for restriction endonuclease XbaJ, a stop codon, and a sequence coding 5 C-terminal amino acids of laminin-binding protein:

5′—T—AAG—TCT—AGA—TGC—ATT—AAT—
TAC—TTA—AGA—CCA—GTC—AGT—GGT—3′
[1].

These oligonucleotides were renatured with plasmid pLBP2, containing a complete copy of the test gene. A DNA fragment containing a full-scale copy of the DNA of the test gene, required for cloning in the expression vector, was obtained by means of a polymerase chain reaction. After elution from the gel and restriction, this fragment was ligated with the vector pATH3. The recombinant plasmid was transformed into strain XL Blue. The clones thus obtained were tested and all contained an insert of the required size — the plasmid was then retransformed into strain CAG-456 [3]. This strain has a mutation in the htpR locus, controlling the heat-shock response. It has been shown that cells with this mutation are defective as regards protein degradation.

Hyperexpression of the component protein was obtained on induction of the Trp-promoter by IAA (Fig. 1). The LBP-TrpE accounted for about 40% of the total soluble protein. The chimera protein is a protein with mol. wt. of 70 kD. After the procedure of isolation and purification this product was used to examine its functional role, namely whether it can or cannot interact with laminin.

The use of immunoblotting to determine specific binding with other proteins is based on the assumption that after gel electrophoresis and transfer to a nitrocellulose filter (NCF) the protein can preserve not only its antigenic determinants, but also its active binding sites. Although this assumption is not always valid, an evident advantage of the method is that it is unnecessary to purify the protein concerned and to identify the required protein on the basis of its mobility in gel. We therefore decided to use this method for the component LBP-protein. To analyze binding with laminin, hybrid protein and TrpE-protein, isolated from cells containing the expressing vector without an insert, coding for the test protein, were applied to neighboring lanes on SDS-PAG. After electrophoretic separation the proteins were transferred to a nitrocellulose filter, and the filter was incubated with laminin. Binding of laminin was then tested with the aid of antibodies to laminin. The antibodies reveal the component protein but do not bind with the induced TrpE product. However, the hybrid protein, interacting with laminin, can also be found in noninduced cells, pointing to incomplete repression of its synthesis. AB to laminin also reveal certain antigenic determinants of *E. coli*.

By ELISA it is possible to analyze binding of a protein product immobilized on a substrate. In this case, by varying the concentration of the substrate, the binding constant of the protein with the substrate can be determined. Another important advantage of this method is absence of any preliminary electrophoretic separation of the proteins, so that they can be hoped to be intact. However, when crude lysates are used, it is difficult to exclude nonspecific binding. To determine the efficiency of binding of the component LBP-TrpE protein with laminin, we covered "Microtiter" plates with the component protein, as described in "Experimental Method." After washing, the plates were incubated with laminin in

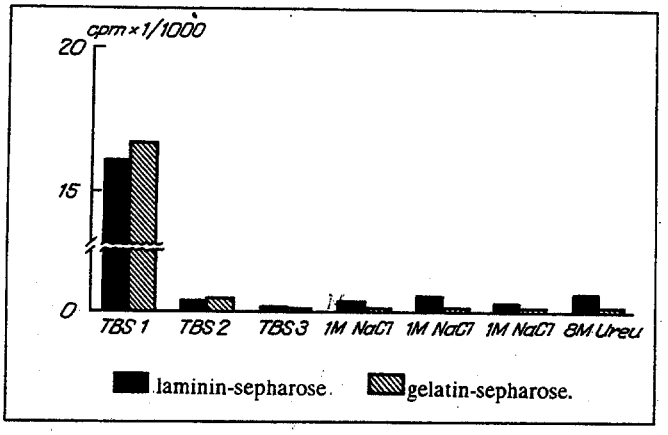

Fig. 3. Analysis of binding of hybrid protein TrpE-LBP with laminin by affinity chromatography. S^{35}-protein was incubated with laminin-sepharose and gelatin-sepharose.

different concentrations. After incubation with antibodies to laminin bound AB were detected with the aid of alkaline phosphatase. The intensity of staining was estimated by spectrophotometer. Examination of Fig. 2 shows that binding of the component protein with laminin was not detected if the laminin concentration was below 100 ng/ml. Within the concentration range from 100 ng/ml to 10 μg/ml effective binding was observed. With laminin in a concentration above 10 μg/ml the level of binding flattens out on a plateau. To rule out the possibility of nonspecific binding, a similar experiment was carried out with plates covered with bovine serum albumin (BSA). It will be clear from Fig. 2 that BSA does not exhibit laminin-binding activity within the concentration ranges of laminin studied.

We also tested binding of LBP-TrpE protein with laminin by affinity chromatography. After preparation of the labeled lysates from induced and noninduced cells, containing the vector of expression, the labeled chimera protein was incubated in parallel experiments with laminin-sepharose 4B (experiment) and gelatin-sepharose 4B (control). Radioactivity was then counted in samples collected after stepwise elution with TBS, 1 M NaCl, and 8 M urea. The results of this experiment are shown in Fig. 3. The diagram shows that most of the label is eluted from the column with TBS. This was observed both with laminin- and with gelatin-sepharose. However, after elution with 1 M NaCl the labeled protein was detected only by laminin-sepharose. Thus the protein binds specifically with laminin-sepharose and is eluted from it with 1 M NaCl. Equivalent amounts of each sample after elution from laminin-sepharose were applied to SDS-PAG for autoradiography. In the case of elution with 1 M NaCl, a major band corresponding to the component protein is visible on the autoradiograph.

It can thus be concluded that the product of the gene which we cloned, and which exhibits homology with the gene of the laminin receptor with molecular weight of 67 kD described previously, can bind with laminin, i.e., post-translation modifications which may arise do not affect binding with laminin. However, it is not yet clear whether LBP 32/67 kD is really a receptor for laminin or whether it binds laminin when discarded from the cell. The biological role of laminin-binding protein requires further study.

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