Ubiquitin-Activating Enzyme (E1) Localised in Human Placenta

UBİKUİTİN AKTİVE EDİCİ ENZİM (E1) İNSAN PLASENTASINDA LOKALİZE OLMUŞTUR

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Summary

As the first enzyme in the ubiquitin (Ub) system, the ubiquitin-activating enzyme (El) plays a pivotal role in all pathways of protein ubiquitination. El was purified from human placenta, and its tissue distribution was investigated. The enzyme was purified by diethyl amino ethyl cellulose (DEAE-cellulose) and affinity chromatography from the material in-question. The purity of the purified El enzyme was tested by sodium dodecyl sulfdte-polyacrylanude gel electrophoresis (SDS-PAGE). An antibody against El enzyme was produced in rabbits for the use in iinmunohistochemical studies. The specificity of the antibody produced was tested with enzyme-linked immunosorbent assay (EL1SA). Tissue sections were stained immunohistochemically with the antibody produced for the investigation of the presence of El enzyme in placental sections. It was concluded that El enzyme is present in placental tissue, since some of the decidual cell cytoplasms showed staining. That El enzyme is localised in human placenta may indicate multiple roles of ubiquitination pathway in this tissue.

Key **Words:** Ubiquitin activating enzyme. Placenta

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Özet

Ubikuitin (Ub) sisteminde ilk enzim olan ubikuitin aktive edici enzim (El) protein ubikuitinasyomınun bütün yollarında temel rol oynar. El insan plasentasından saflaştırıldı ve doku dağılımı araştırıldı. Enzim material içeriğinden afinite kromatografisi ve dietil-amino-etil selüloz (DEAE-selüloz) ile saflaştırıldı. Saflaştırılmış El enziminin saflığı sodyum dodesil sülfatpoliakrilamidjel elektroforezi ile (SDS-PAGE) test edildi. İmmunohistokiınyasal çalışmalarda kullanmak için El enzimine karşı antikor tavşanlarda üretildi. Üretilmiş antikorun spesifitesi enzim-linked immunosorbent metodu (ELİSA) ile test edildi. Plasenta kesitlerinde El enziminin varlığını araştırmak için doku kesitleri üretilmiş antikor ile immunohistokiınyasal olarak boyandı.

Desidual hücre sitoplazmalarının bazıları boyandığından dolayı, El enziminin plasenta dokusunda olduğu sonucuna varıldı. İnsan plasentasında El enziminin lokalize olması bu dokuda ubikuitinasyon yolunun mu/tiple rolünü göstermelidir.

Anahtar Kelimeler: Ubikuitin aktive edici enzim. Plasenta

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Ubiquitination, being a covalent modification of cellular proteins, has a role in a variety of physiological processes, the best understood of which is the ubiquitin-dependent degradative pathway (1). After ubiquitination, the proteins are targeted for degradation. A three-step mechanism for Ub-protcin conjugate formation has been proposed (2). After activation to a thiol ester via tightly bound Ub adenylate by E l , Ub is transferred to thiol groups on a number of low-molecular weight proteins, collectively-termed Ub earner proteins (E2s). One or more of the E2-Ub thiol esters then donates Ub to a protein amino group in a reaction catalyzed by Ub-protcin ligase (E3). Protein molecules can be multiply ubiquitinated even at very low extents of protein reaction, suggesting that Ub transfer may be processivc (3). The first reaction which is catalysed by El in Ub conjugation is the activation of Ub to a high-energy intermediate. The Ub-activating enzyme catalyzes the activation of Ub in the three reversible steps. There are two active sites within the El molecule, allowing it to accommodate two Ub moieties at a time, with a new Ub forming an adenylate intermediate as the previous one is trans-

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ferred to the thiol site (4). The purified enzyme has an apparent Mr=210 kDa and appears to be composed of two subunits of Mr=105 kDa (5). In our early study, we have found that ethilmaleimid, EDTA and mercurynitrate were inhibitors of the enzyme, optimum pH of the enzyme was 7 and optimum temperature was 35°C (6).

In the literature review related to this subjects, it was found that El enzyme was studied in yeast (7), wheat (8) and some tissues of human being such as blood, skeleton and lungs (9,10). To the best of our knowledge, we could not find any investigation related to placenta. In this study, therefore, we aimed to investigate whether El is present in placenta and the presence of cellular protein degradation in this tissue which provides a relation between the mother and the fetus.

Materials and Methods

Human El:Ub-activating enzyme was purified from placenta according to Ciechanover method of purification of Ub-activating from erythrocytes and reticulocytes and according to methods used by Scheider, Cuatrecases, and Kohn (11-15). The placenta (weighing 700 gr) was washed three times with 150 mM KC1 +200 uX mercaptoethanol solution. This tissue was divided into four equal parts (each 175 gr) and then was homogenised in 20 mM Tris-HCl +1 mM EDTA and 2% glycerol solution (30v/5v/10v) with previously cooled cycle blend homogenisator (Fisher). The homogenate was centrifuged at 13000 x g and the supernatant was collected. This 200 mL supernatant was incubated in 150 mL 0.2 mM 2,4-dinitrophenol and 150 mL 20 mM 2-deoxyglocose solutions for 2 hours at 37°C; then it was treated with 1 mM 2 L DTT solution and centrifuged at 80 000 x g for 90 minutes and the supernatant was taken (fraction I). Sixty mL of supernatant was loaded onto 3x74 cm DEAE-cellulose (Whatman, DE-52) which was pretreated with 3 mM K_9P0_4 (pH=7). Unbounded molecules were eluted with 150 mL 3 mM K_2P0_4 containing 1 mM DTT After that, the bounded molecules were eluted with the help of 150 mL eluting solution containing 0.5 mM KC1+1 mM DTT and 10 mM Tris-HC1 (pH=7.2). After precipitation of the eluate with $(NH_4)_0SO_4$ on dialysis, the solution was collected (fraction II). Ten gr of sepharose 4B complex was swollen with distilled water and the remaining water was poured. Four hundred mL Ub (Sigma) functioning as a ligand, 1 mg a 6-carbon N-hydroxysuccinamide (Sigma) as a spacer ami, 200 mL glacial acetic acid, 800 mL Tris-HCl (pH=7.2) and swollen sepharose 4B were mixed with a stirrer at a low rate over night at +4°C. The homogenous mixture obtained was packed into columns (Pharmacia, 2x15 cm) and was washed three times, each time using 0.1 M Na-acetate (pH=4) containing 1 M NaCl and 0.1 M Tris-HCl (pH=8) solutions, respectively. The column was balanced with a buffer solution [0.2 mM DTT+5 mM $MgCl_2$+2 mM ATP and 50 mM Tris-HCl (pH=7.2)]. Thirty mL of fraction II solution was mixed with 50 mM Tris-HCl, 5 mM ATP, 10 mM $MgCl_2$, 0.2 mM DTT and 5 U/mL inorganic pyrophosphatase (Sigma) and this homogenous solution obtained was loaded onto the column. The column was washed with the buffer solution above followed by 50 mM Tris-HCl (pH=7.2) containing 1 M KC1. This procedure was repeated 3 times. The bounded molecules were eluted with the aid of 50 mM Tris-HCl (pH=7.2) solution containing 2 mM AMP (Sigma) and 0.04 mM NaPPj (Sigma). Protein concentration was determined by the method of Lowry et al. (16) using bovine serum albumin as standard. The purity of the enzyme was tested with SDS-PAGE (17).The protein contents of fraction II solution and eluate obtained from the column (E1 protein) were found as 25-30 mg/mL and 3-5 mg/mL, respectively.

Anti-El mAbs: For obtaining mAbs to human E1, rabbits were subcutanously injected with 50 |ig of purified El protein in 100 uX acetic acid, 800 mL Tris-HCl (pH=7.2) and 1 mL Freund's adjuvant (Sigma). This procedure was repeated three times in a 20-day period. Twenty five days after the last injection, blood samples were taken and incubated at 37°C for one night, then the fust rnAb in serum obtained was used in immunohistochemical studies. The specificty of anti-El mAb was tested by ELISA.

Immunohistochemical study: Immunohistochemical studies were performed according to peroxidase-antiperoxidase labelling method (18). Tissue sections with the thickness of 6-7 mm fixed on the lamel were incubated in 80% xylene and 90% ethanol solutions. Then, incubation was car-

ried out in peroxidase inhibiting solution (2% H_2O_2+60% methanol) for 20 minutes. After being washed with water, tissue sections were incubated in 100% fonnic acid solution for 3 minutes and in blocking solution (10% fetal calf serum+0.15 M NaCl+0.01 M Na_2HP0_4, pH=7.2) at 4 °C for 5 minutes, respectively. One hundred U.L of anti-El mAbs (1:500 dilution) was added on tissue sections and incubated at 4°C for 15 hours. After washing, 100 uX of peroxidase conjugating second mAb (1:200 dilution, Sigma) was added on tissue sections and incubated at 4°C for 15 hours. The dilutions of mAbs were performed with the 10% phosphate buffered saline solution (10 mM Na_2PO_4+0.9% NaCl, pH=7.2). After washing process, incubation was continued in staining solution (0.15% NaCl+0.01 M NajHPO^O.1S gr 3,3'-diaminobenzidin+0.2 gr imidazole+45 U.L H_20_2, pH=7.2) for 10 minutes and washing was done with 0.5% $CuSO_4$+0.9% NaCl. Following a ten-minute incubation in 70% hemotoxyline solution, the tissue sections were incubated in acid-alcohol solution (0.5% HCl+70% ethanol) for 10 minutes. After being washed with tapering water, tissue sections were passed through 50%, 95%, 100% ethanol solutions and 80%) xylene solutions for 3 minutes each, respectively. Tissue sections were dyed and their photographs were taken.

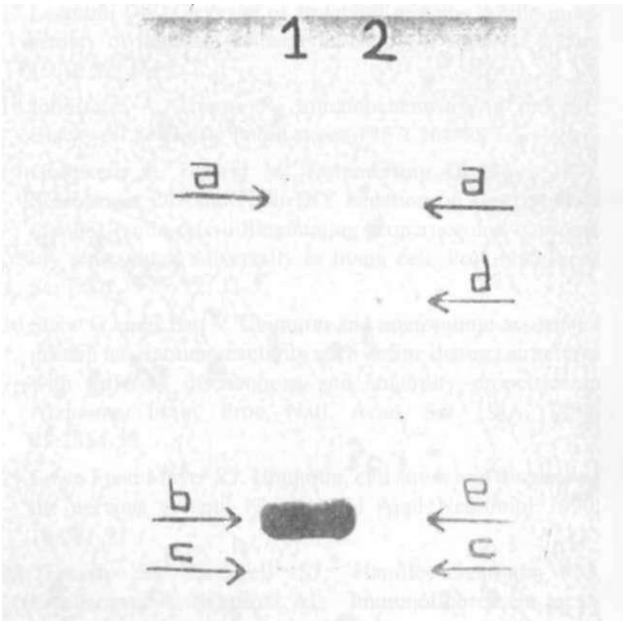

Figure 1. Purified human placenta E1. Coomassie blue stain of 14% SDS/PAGE analysis of human El purified from placenta.
Lane 1: a) Catalase (230 kDa)*, b) Purified E1, c) Phosphorylase b (92,5 kDa)*;
Lane 2: a) Catalase (230 kDa), d) Myosin (200 kDa)*, e) b-Galactosidase (116 kDa)*, c)Phosphorylasc b
*Molecular markers (Sigma).

Results and Discussion

Recent studies have shown that the conjugation of Ub with proteins may play an important role in the energy-dependent degradation of intracellular proteins (19). It is possible that disruption of the cytoskcleton in neurodegenerative disorders by improper localization of Ub system components or the appearance of aberrant protein conjugation could lead to altered proteolytic processing and contribute to the pathology of neurological disease. Indeed, Ub conjugates have been found to be associated with neurofibrillary tangles of Alzheimer's disease (20), Lewy bodies in Parkinson's disease, and Pick's bodies in Pick's disease (21). Both El and Ub conjugates colocalize with actin fibers, intermediate filaments, and microtubules (1).

El was >95% purified from human placenta via immobilized Ub affinity chromatography yielding a single polypeptide of -105 kDa after

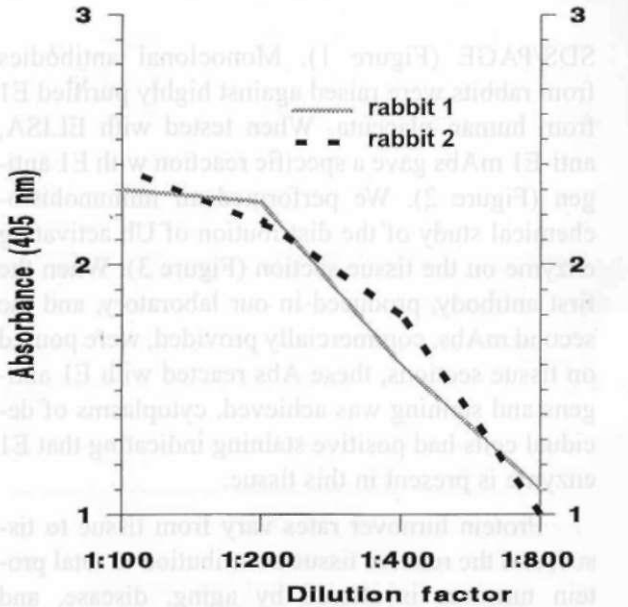

Figure 2. Results of specificity of mAbs produced in rabbits.

Figure 3. Immunohistochcmical localisation of E1. As mentioned in Materials and Methods section, the tissue was fixed and dyed, (x 400).

SDS/PAGE (Figure 1). Monoclonal antibodies from rabbits were raised against highly purified El from human placenta. When tested with ELISA, anti-El mAbs gave a specific reaction with El antigen (Figure 2). We performed an immunohistochemical study of the distribution of Ub activating enzyme on the tissue section (Figure 3). When the first antibody, produced in our laboratory, and the second mAbs, commercially provided, were poured on tissue sections, these Abs reacted with El antigens and staining was achieved, cytoplasms of decidual cells had positive staining indicating that El enzyme is present in this tissue.

Protein turnover rates vary from tissue to tissue, and the relative tissue contribution to total protein turnover is altered by aging, disease, and changes in dietary protein intake. Several proteins have short turnover times, sometimes less than 1 hour. During periods of growth, pregnancy, lactation, or recovery from illness, protein requirement increases. Trausch et al (22) showed the localisation of El enzyme both in cytoplasmic and in nuclear compartments of some different eukaryotic cells (HeLa, Smooth muscle A7r5, choriocarcinoma BeWo, PtK1, and Chinese hamster ovary (CHO) E36). In addition, they also found that this enzyme was associated with actin filaments, tubulin and intermediate filaments in cytoplasms of CHO and ptK1 cells. Cook and Chock (23) have described El as being concentrated in nuclei of rat brain and liver. In our study, cytoplasms of decidual cells showed positive staining. The variable distribution of El in different cell types including placenta, including its apparent cytoskelctal association, suggests pleiotropic functions of this enzyme and the Ub-conjugating system (22-24).

Thus, we have defined the immunolocalization of El in human placenta, within cytoplasm of decidual cells. It is possible that El and other components of the Ub system may play distinct, essential roles by localizing different subcellular compartments within the cell. As a result, this study showed that El enzyme is present in placental tissue. Further studies are required to clarify the relation Ub-activating enzyme and placental state.

REFERENCES

1. Finlcy D and Chau V. Ubiquitination. Annu Rev Cell Biol 1991; 7:25-69.
2. Hershko A, Heller H, Elias S and Ciechanover A. Components of Ubiquitin-protein ligase system. .1 Biol Chem 1983; 258: 8206-14.
3. Hershko A, Ciechanover A, Heller H, Haas A L, Rose IA. () Proposedrole of ATP in protein breakdown Conjugation of proteins with multiple chains of the polypeptide of ATP-dependent proteolysis: Proc Natl Acad Sci 1980; 77:1783-86.
4. Haas A L, Rose IA. The mechanism of Ubiquitin activating enzyme. J Biol Chem 1982; 257:10329-337.
5. Ciechanover A, Elias S, Heller H, Hershko A. Covalent affinity purification of Ubiquiting-activating enzyme. The J Biol Chem 1982; 257:2537-42.
6. Kiziltunc A, Pirim I, Sahin Y N. Obtaining of ubiquitin activating enzyme and kinetic features. T Clin J Med Sci 1995; 15:260-4.
7. McGrath JP, Jentsch S, Varshavsky A. UBA 1 an essential yeast gene encoding ubiquitin-activating enzyme. EMBO J 1991; 10(l):227-36.

8. Hatfield P M , Callis J, Vierstra R. () Cloning of ubiquitin-activating enzyme from wheat and expression of a functional protein in Escherichia coli. J Biol Chem 1990; 265:15813-17.

9. Handley P M , Mueckler N, Siegel R, Ciechanower A, and Schwartz A L . Molecular cloning, sequnce, and tissue distribution of the human ubiquitin-activating enzyme E I . Proc Natl Acad Sci U S A 1991; 88: 258-62.

10. Zacksenhaus E, Shcinin R. Molecular cloning primaiy structure and expression of the human X linked A1S9 gene c D N A which complements the ts A1S9 mouse L cell defect in D N A replication. E M B O J 1990; 9(9):2923-29.

1 l.Scheider R, Eckerskon C, Lottspeich F and Schweiger M. The human ubiqutin carrier protein E2 (17kDa) is homologous to the yeast D N A repaire gene, R A D 6 . E M B O J 1990; 9(5): 1431-35.

12. Cuatrecases P, Wilchek M, Anfinsen C B . Selective enzyme purification by affinity chromatoghraphy. Proc Nat Acad Sci 1968; 61: 636-43.

13. Kohn J, Wilchek M. A colorimetric method for monitoring activation of sepharose by cyanogen bromide. Biochem * Biophy Res Commun 1978; 7-14.

14. Ciechanover A, Elias S, Heller H and Hershko A. Covalent affintiy purification of ubiquitin-activating enzyme. J Biol Chem 1982; 257:2537-42.

15. Cicchanover A, Hod Y, Hershko A. A heat-stable polypeptide of an ATP-dependent proteolytic system from reticulocytes. Biochem Biophy Res Commun 1978; 81(4): 1100-05.

16. Lowry O H , Roscbrough N J . Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265.

17. Laemmli D K . Cleavage of structural proteins during in assembly of the head of bacteriophage T4, Nature London. 1970: 227: 680.

18. Johnstone A, Thorpe R. Immunochemistry in practice. Blackwell Scientific Publications 1987; 261-88.

19. Goldstein G, Scheid M, Hammerling U, Boyse E A , Schlesinger DH and Niall H D . Isolation of a polypeptide that has lymhocyte-differentiating properties and is probably represented universally in living cell. Proc Natl Acad Sci U S A 1975; 72: 11-5.

20.Shaw G and Chau V. Ubiquitin and microtubulc-associated protein tau immunoreactivity each define distinct structures with differing distributions and solubility properties in Alzheimer brain. Proc. Natl. Acad. Sci. U S A . 1988; 85:2854-58.

21. Lowe J and Mayer RJ. Ubiquitin, cell stress and diseases of the nervous system. Neuropathol Appl Ncurobiol 1990; 16:281-91.

22. Trausch JS, Grenfell SJ, Handley-Gearhart P M , Ciechanover A, Schwartz A L . Immunofluorescent localization of the ubiquitin-activating enzyme, E l , to the nucleus and cytoskeleton. American Physiological Society 1993; 93:93-102.

23. Cook JC, Chock P B . Immunocytochemical localization of ubiquitin-activating enzyme in the cell nucleus. Bioch BiophRes Com 1991; 174(2): 564-71.

24.Schwartz A L , Trausch JS, Ciechanover A, Slot JW, Geuze H. () Immunoelectron microscopic localization of the ubiquitin-activating enzyme El in HepG2 cells. Proc Natl Acad Sci 1992; 89: 5542-44.