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Molecular Cloning and Expression of *Caenorhabditis elegans* ERp57-Homologue with Transglutaminase Activity[1]

Shunji Natsuka,[2] Ritsuko Takubo, Ryo Seki, and Koji Ikura

Department of Applied Biology, Faculty of Textile Science, Kyoto Institute of Technology, Kyoto 606-8585

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Formation of cross-linking between proteins *via* a γ-glutamyl-ε-lysine residue is an important process in many biological phenomena including apoptosis. Formation of this linkage is catalyzed by the enzyme transglutaminase, which is widely distributed from bacteria to the animal kingdom. The simple multi-cellular organism *Caenorhabditis elegans* also possesses transglutaminase activity associated with apoptosis [Madi, A. *et al.* (1998) *Eur. J. Biochem.* 253, 583–590], but no gene with significant homology to vertebrate or bacterial transglutaminases has been found in the *C. elegans* genome sequence database. On the other hand, protein disulfide isomerases were recently recognized as a new family of transglutaminases [Chandrashekar, R. *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95, 531–536]. To identify the molecule with transglutaminase activity in *C. elegans*, we isolated from *C. elegans* a gene homologous to ERp57, which encodes a protein disulfide isomerase, expressed it in recombinant form, and characterized the transglutaminase and protein disulfide isomerase activities of the resultant protein. The *C. elegans* ERp57 protein had both enzyme activities, and the transglutaminase activity had similar characteristics to the activity in lysate of the whole worm. These results suggested that the ERp57 homologue was one of the substances with transglutaminase activity in *C. elegans*.

Key words: *Caenorhabditis elegans*, cloning, ERp57, transglutaminase.

Transglutaminases catalyze the cross-linking of proteins through γ-glutamyl-ε-lysine isopeptide in a calcium-ion-dependent manner. The enzymes constitute a family of closely related thiol enzymes that are derived from a common ancestor (*1*). Five enzymatically active transglutaminases have been identified in mammals: keratinocyte-type, tissue-type, epidermal-type, prostate-type, and subunit A of clotting factor XIII. Recent reports have demonstrated the relationship between transglutaminase activity and various stages of apoptosis (*2–4*). The molecular mechanism of programmed cell death in mammalian cells has not been completely clarified, but much relevant information has been obtained from *Caenorhabditis elegans*, a model organism widely employed in developmental genetics. A number of genes involved at various steps of this process in *C. elegans* have been identified, and some of their mutants showed lower transglutaminase activity than the wild type in lysates of the worm (*5*). To elucidate the role of the enzyme activity in apoptosis, we planned to isolate a gene for transglutaminase from *C. elegans*. Although the enzyme activity was found in the worm lysate, however, no significant homologue of authentic mammalian or bacterial transglutaminase genes appears in the worm genome. Makarova *et al.* have reported extremely limited homology of the bacterial and mammalian transglutaminase sequences around their catalytic sites (*6*). They also pointed out that a putative protein of *C. elegans* (GenBank accession No. CAA-87787) had slight similarity to their consensus sequence. However, none of the three amino acid residues constituting the catalytic triad was maintained in the worm sequence. Thus, they concluded that a protein derived from the sequence of *C. elegans* would probably be inactive, and could not account for the transglutaminase activity in the worm lysate reported by Madi *et al.* (*5*). Recently, Chandrashekar *et al.* clearly demonstrated that the *Dirofilaria immitis* protein disulfide isomerase ERp60 (which is the same as ERp57), also had transglutaminase activity (*7*). These findings suggested the possibility that transglutaminase activity in *C. elegans* was due to a homologue of ERp57 protein. From this point of view, we examined the transglutaminase activity of the ERp57 homologue from *C. elegans*.

MATERIALS AND METHODS

Molecular Cloning from C. elegans—Mixed cultures of *C. elegans* (Bristol N2) were collected from culture plates and washed with M9 buffer to remove bait. Total RNA of the worm was prepared by use of a Quick Prep total RNA extraction kit (Amersham Pharmacia Biotech). The cDNA of the ERp57 homologue (ceERp57) was isolated by RT-PCR with a ReverTra Dash kit (Toyobo). The PCR primers had the sequences AGGTAATGATTTGGGTCCAGGCAG and TTATCCAATTACAATTCAGTCTTTC. The cDNA was sub-

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[2] To whom correspondence should be addressed. E-mail: natsuka@ipc.kit.ac.jp

cloned into pGEM-T Easy Vector (Promega), and its structure was confirmed by sequencing with ABI Prism 310 DNA analyzer (Applied Biosystems).

Alignment of Amino Acid Sequences and Construction of a Phylogenetic Tree—The amino acid sequences of ERp57 proteins and protein disulfide isomerases were obtained from the GenBank/EMBL/DDBJ database. Neighbor-joining analysis (*8*) of the distance matrix calculated from amino acid sequence alignment provided the phylogenetic tree.

Gene Expression and Purification—A *Xho*I-*Sal*I fragment of ceERp57 cDNA, which includes the open-reading frame except for the sequence corresponding to the signal peptide, was subcloned into an expression vector, pET19b (Novagen), to construct pET19b-ceERp57. The origin of the *Sal*I site of the fragment was from pGEM-T Easy Vector. The expression vector was inserted into BL21(DE3) strain of *E. coli*, and its expression was induced with 0.4 mM IPTG at 25°C. The bacteria were lysed by sonication in buffer of 20 mM Tris-HCl (pH 7.9), 5 mM imidazole, 0.5 M NaCl. The soluble materials, isolated by centrifugation and filtration, were loaded onto Ni^{2+}-chelating resin column. The column was washed with 20 mM Tris-HCl (pH 7.9), 60 mM imidazole, 0.5 M NaCl, then bound materials were eluted with 20 mM Tris-HCl (pH 7.9), 1 M imidazole, 0.5 M NaCl. The eluate was dialyzed against 0.1 M Tris-HCl (pH 8.0), and protein concentration was measured by micro BCA protein assay reagent (Pierce).

Enzyme Assays—The transglutaminase activity was measured by the microtiter plate assay with *N,N'*-dimethylcasein as an amine-acceptor substrate and 5-(biotinamide)-pentylamine as an amine-donor substrate (*9*). Protein disulfide isomerase activity was measured by refolding of "scrambled" RNase, which had exchanged cystines, as described elsewhere (*10*). Bovine protein disulfide isomerase (Sigma) was used as a positive control of the enzyme.

RESULTS

Molecular Cloning of ERp57 Homologue from C. elegans—Homology search of mammalian and *Dirofilaria immitis* ERp57 (ERp60) genes in the *Ceanorhabditis elegans* genome database yielded the H06O01.1 gene as the putative homologue. The cDNA was prepared from *C. elegans* (Fig. 1) and designated as the ceERp57 gene. The open-reading frame was predicted to be a polypeptide of 488 amino acids. Hydropathy analysis (*11*) indicated that it had single hydrophobic region in its N-terminal portion. Its amino acid sequence shares 61 and 43% identity with *Dirofilaria immitis* and human ERp57s, respectively (Fig. 2A). To predict the evolutional distances of ceERp57 and related gene sequences of *C. elegans*, we drew up a phylogenetic tree for the ERp57 orthologues and protein disulfide isomerases (Fig. 2B). Only ceERp57 was included in the ERp57 family, whereas other four related sequences from the *C. elegans* genome database were included in the pro-

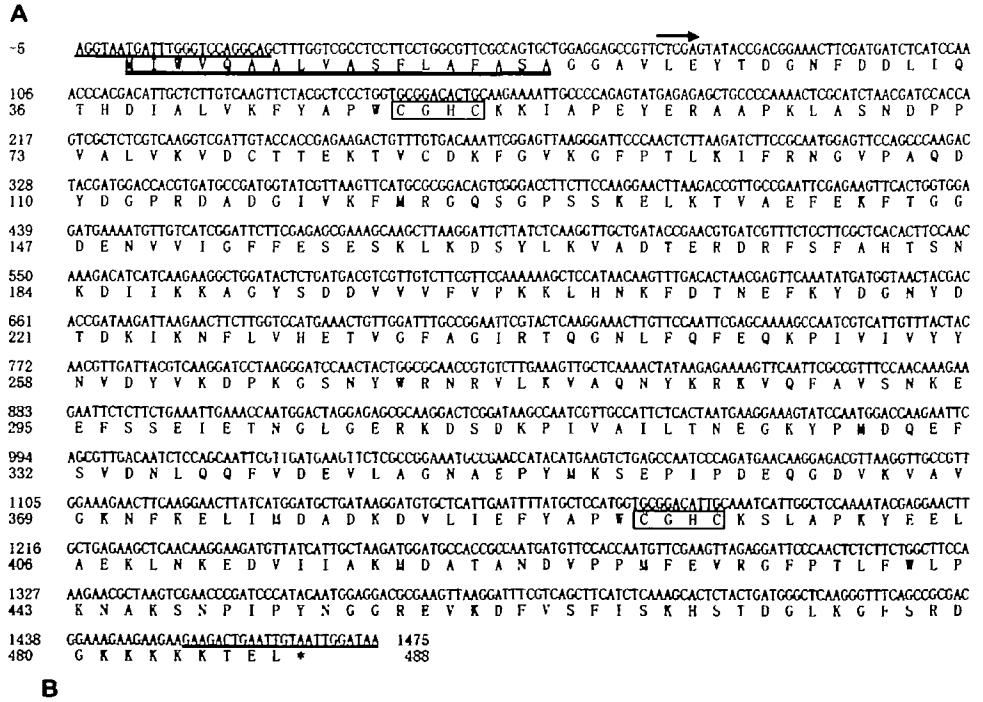

Fig. 1. **Nucleotide and deduced amino acid sequences of ceERp57 gene.** A: Nucleotide and amino acid sequences of ceERp57. The adenine residue of the putative initiation codon is assigned as residue 1 of the ceERp57 nucleotide sequence. The methionine residue corresponding to this putative initiation codon is assigned as residue 1 of deduced amino acid sequence. The amino acid sequence of the predicted signal peptide is double-underlined. The DNA sequences included in PCR primers for the cloning are single-underlined. An arrow indicates the position of the *Xho*I site used for the recombinant preparation. Boxes indicate the positions of consensus sequences of thioredoxin-active sites. B: Hydropathy plot of the predicted ceERp57 polypeptide. The plot was calculated by the method of Kyte and Doolittle (*11*).

A

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ceERp57     -------MIWVQAALVASFLAFASAGGAVLEYTDGNFDDLI---QTHDIALVKFYAPWCGHCKKIAPEYERAAPKLASNDPPVALV
DiERp57     MTLVRLFDASIFKLFLFLI*PLTN*D*D*MKF**AD*KEG*---KPY*VL*****************F*K**T**LQ****IH*A
hERp57      --MRLRRLALPPGVALLLAA*RLA*ASD***L**D**ESR*SDTGSAGLM**E*F********RL*****A**TR*---KGI*P*A

ceERp57     KVDCTTEKTVCDKFGVKGFPTLKIFRNGVPAQDYDGPRDADGIVKFMRGQSGPSSKELKTVAEFEKFTGGDENVVIGFFESE-SKL
DiERp57     E****E**KT**EY**S*********K*EL********V*E****Y****A***AT*IN*QQ****MLQA*DVTIC****EN-***
hERp57      *****ANTNT*N*Y**S*Y*******D*EE*GA*****T*****SHLKK*A**A*VP*R*EE**K**ISDKDASIV***DDSF*EA

ceERp57     KDSYLKVADTERDRFSFAHTSNKDIIKK-AGYSDDVVVFVPKKLHNKFDTNEFKY-DGNYDTDKIKNFLVHETVGFAGIRTQGNLF
DiERp57     ***F***********K*VW****Q*LES-R**N**I*AYQ***F****EP*****-**********E**L***N*LV****AE*RY
hERp57      HSEF**A*SNL**NYR****NVESLVNEYDDNGEGIIL*R*SH*T***EDKTVA*TEQKMTSG***K*IQENIF*ICPHM*ED*KD

ceERp57     QFEQKPIVIVYYNVDYVKDPKGSNYWRNRVLKVAQNY---KRKVQFAVSNKEEFSSEIETNGLGERKDSDKPIVAILTNEG-KYPM
DiERp57     *YDLL*MFV**GK***EL************M**KD*---***AN**M****D**FDLDEF**AN***T-**L**ARSKK*-*FF*
hERp57      LIQG*DLL*A**D***E**A**********MM**KKFLDAGH*LN***ASRKT**H*LSDF**-*STAGEI*V***R*AK*E*FV*

ceERp57     DQE--FSVDNLQQFVDEVLAGNAEPYMKSEPIPD-EQGDVKVAVGKNFKELIMDADKDVLIEFYAPWCGHCKSLAPKYEELAEKLN
DiERp57     KE*FS***E**KK**ED*IGDRL******EA*E-D******V*A*T*Q*M**NVE***************A*****D**GQ**S
hERp57      QE*FSRDGKA*ER*LQDYFD**LKR*L******ESND*P***V*AE**D*IVNNEN***************N*E***K**G***S

ceERp57     KE-DVIIAKMDATANDVPPMFEVRGFPTLFWLPKNAKSNPIPYNGGREVKDFVSFISKHSTDGLKGFSRDGKKKKKTEL
DiERp57     G*PG*V*************P*Q*Q*****Y*V***K*DK*E**S*****D**IKY*A**A*EE***YK****P***E**
hERp57      *DPNIV************SPYE******IYFS*A*K*L**KK*E****LS**I*YLQREA*NPPVIQEEKP*****AQEDL
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B

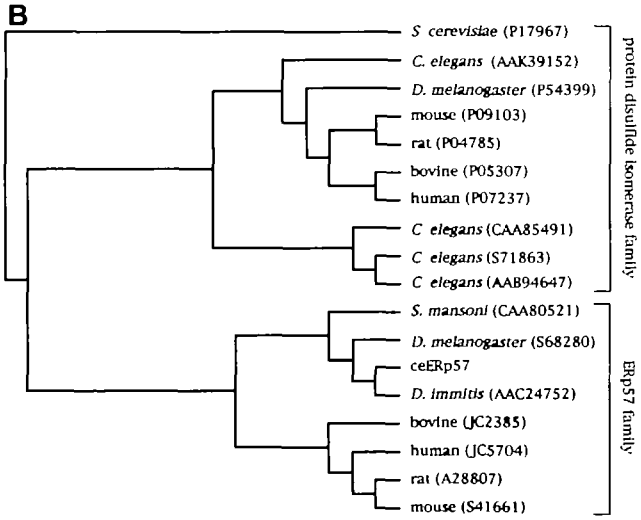

Fig. 2. **Amino acid homology between ERp57s and related proteins.** A: The deduced amino acid sequences are indicated by the one letter notation. Amino acid residues in *D. immitis* (*7*) and human (*12*) ERp57s identical to those in *C. elegans* ERp57 are shown by asterisks. Gaps are indicated by dashes. Underlines indicate the positions of consensus sequences of thioredoxin-active sites. B: Phylogenetic tree of the ERp57 proteins and protein disulfide isomerases. Accession numbers of each amino acid sequences in the GenBank database are indicated in parentheses.

tein disulfide isomerase family.

Expression of the ERp57 Homologue in E. coli—The cDNA of ceERp57 excluding the putative signal peptide sequence was inserted into a bacterial expression vector to produce the recombinant protein in *E. coli*. The recombinant ceERp57 including an artificial oligo-histidine sequence was expressed as soluble material and purified using a nickel-chelating column (Fig. 3). The isolated recombinant protein was purified to homogeneity by electrophoresis and used in further experiments.

Protein Disulfide Isomerase Activity Assay—To confirm whether ceERp57 had a protein disulfide isomerase activity like the other ERp57 orthologues, the enzyme activity of ceERp57 protein was assayed by measuring the refolding of "scrambled" RNase (Fig. 4A). The recombinant ceERp57 was able to repair the exchanged cysteine connections of RNase at a similar rate to bovine protein disulfide isomerase. The enzyme activity was also found in whole worm lysate (Fig. 4B).

Transglutaminase Activity Assay—Transglutaminase activity was evaluated by the introduction of 5-(biotinamide)pentylamine into *N,N'*-dimethylcasein by transamidation. The recombinant ceERp57 catalyzed the transamidation in a time- and protein-concentration-dependent manner (Fig. 5). Optimal temperature was 55°C (Fig. 6), and optimal pH was 8.0 (Fig. 7). To examine the similarity of transglutaminase activities of recombinant ceERp57 and whole worm lysate, the effects of various reagents on their activities were assayed (Table I). Both activities were dependent on calcium ions and were abolished by addition

of EDTA. Inhibitors of vertebrate transglutaminases, ammonium chloride, iodoacetamide, and mercury ions, only moderately decreased both enzyme activities.

DISCUSSION

We isolated from *C. elegans* a gene homologous to ERp57, which encoded a protein disulfide isomerase. The deduced amino acid sequence includes two thioredoxin family active-site sequences (CGHC) that are involved in disulfide bond formation and rearrangement reactions. The gene expressed an enzyme that has activity of exchanging disulfide bonds in "scrambled" RNase, as do other protein disulfide isomerase family proteins. Furthermore, the phylogentic tree of the related sequences indicated that the gene was the most homologous in the *C. elegans* genome to mammalian ERp57 genes. Therefore, we concluded that the gene we cloned from *C. elegans* was an orthologue of ERp57, and we named it ceERp57.

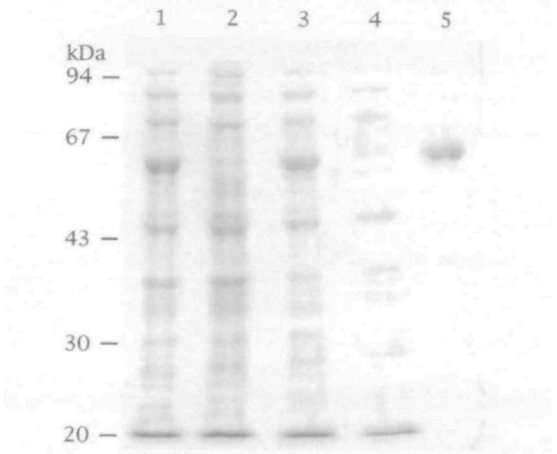

Fig. 3. **Purification of recombinant ceERp57.** SDS-PAGE of samples from each purification step. 1: Whole lysate of *E. coli* carrying the pET19b-ceERp57 plasmid with IPTG induction. 2: Whole lysate of the *E. coli* without induction. 3: Soluble fraction of the lysate from the induced bacteria. 4: Pass-through fraction of Ni^{2+}-chelating resin column chromatography. 5: Eluate of Ni^{2+}-chelating resin column chromatography.

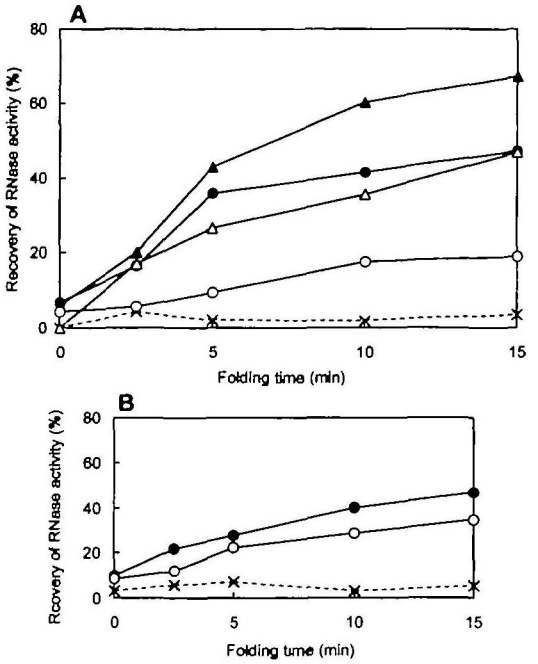

Fig. 4. **Protein disulfide isomerase activity of ceERp57 and the worm lysate.** A: Protein disulfide isomerase activity of ceERp57 and bovine protein disulfide isomerase. Open and filled circles indicate the activity of 10 and 50 μg of the recombinant ceERp57, respectively. Open and filled triangles indicate the activity of 10 and 50 μg of bovine protein disulfide isomerase, respectively. Dashed line with X indicates negative control without enzyme. B: Protein disulfide isomerase activity of the whole worm lysate. Open and filled circles indicate the activity of 50 and 100 μg of the lysate, respectively. Dashed line with X indicates negative control without enzyme.

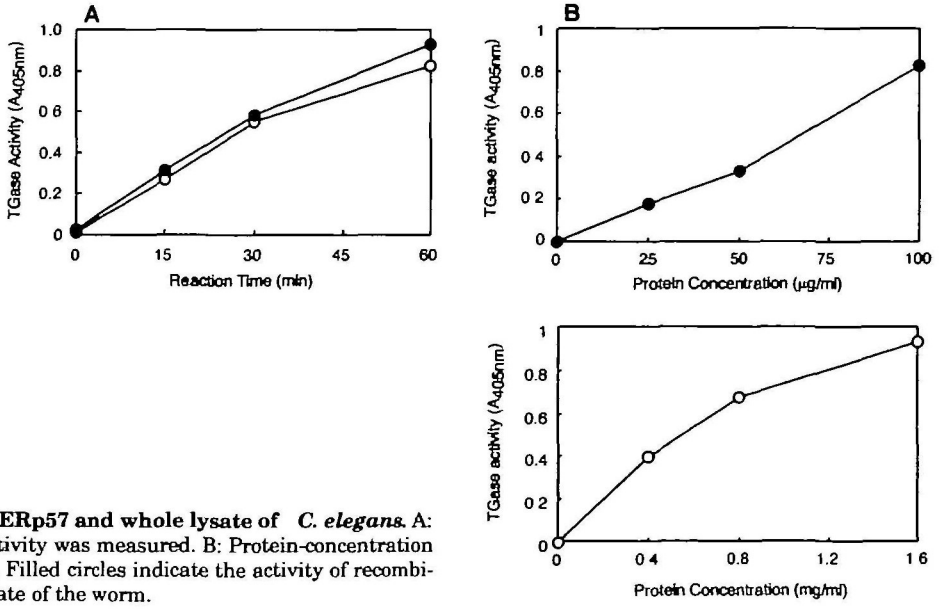

Fig. 5. **Transglutaminase activity of ceERp57 and whole lysate of *C. elegans*.** A: Time-dependency of transglutaminase activity was measured. B: Protein-concentration dependency of the activity was measured. Filled circles indicate the activity of recombinant ceERp57. Open circles are whole lysate of the worm.

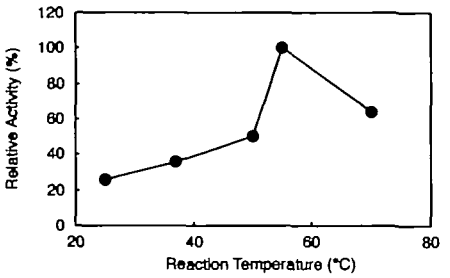

Fig. 6. **Temperature-dependency of transglutaminase activity.** Transglutaminase activity was measured at various temperatures. The values shown are relative activities to that at 55°C as 100%.

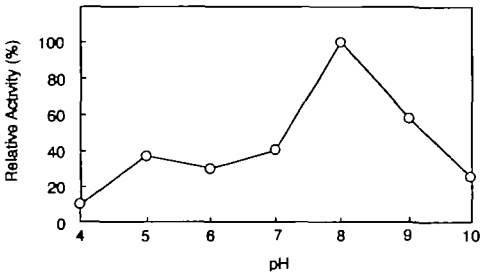

Fig. 7. **The pH-dependency of transglutaminase activity.** Transglutaminase activity was measured at various pHs. The buffers used were 0.1 M sodium acetate (pH 4 and 5), 0.1 M imidazole-HCl (pH 6 and 7), 0.1 M Tris-HCl (pH 8 and 9), and 0.1 M sodium glycine (pH 10). The values shown are relative activities to that at pH 8 as 100%.

The ceERp57 protein also had calcium ion-dependent transglutaminase activity. Optimal pH was 8.0, and optimal temperature was 55°C. In general, enzymes in *C. elegans* are fully active at lower temperature than mammalian enzymes, because this organism lives at below 25°C. Thus, stability for temperature of this enzyme might be extremely high. The recombinant ceERp57 also showed similar susceptibilities to several inhibitors of mammalian transglutaminases to the activity of whole worm lysate. These results suggest that the ceERp57 protein is one of the substances with transglutaminase activity in *C. elegans*.

The purpose of our project including this study is to elucidate the function of transglutaminase in apoptosis. However, whether the ceERp57 protein is involved in apoptosis remains unknown. The study of cross-linking by transglutaminase in cells also remains for the future. Investigations by reverse-genetic approaches involving RNA-interference and gene disruption by transposon are proceeding to clarify the physiological function of the ceERp57 protein.

TABLE I. **Characterization of transglutaminase activity of ceERp57 and *C. elegans* lysate.** The effects of several compounds on transglutaminase activity of ceERp57 and *C. elegans* lysate were determined by a microtiter plate assay (*9*).

Treatment	Activity (%)	
	ceERp57	*C. elegans* lysate
Control ($+Ca^{2+}$)	(100)	(100)
10 mM EDTA	0.1	1.6
5 mM $MgCl_2$ ($+Ca^{2+}$)	56	80
5 mM $MgCl_2$	14	21
5 mM NH_4Cl ($+Ca^{2+}$)	60	66
10 mM iodoacetamide ($+Ca^{2+}$)	29	32
10 mM iodoacetamide	56	52
5 mM $HgCl_2$ ($+Ca^{2+}$)	85	40

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