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Biochemical and Biophysical Research Communications 296 (2002) 54–61

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Nuclear translocation of extracellular superoxide dismutase

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Received 3 July 2002

Abstract

Histochemical examination of mouse tissues showed nuclear staining of extracellular superoxide dismutase (EC-SOD), and the nuclear translocation of EC-SOD was also confirmed in cultured cells that had been transfected with its gene, as shown by immunohistochemistry and Western blot analysis. The EC-SOD which was secreted into the medium was incorporated into 3T3-L1 cells and a significant fraction of the material taken up was localized in the nucleus. Site-directed mutagenesis indicated that the heparin-binding domain of EC-SOD functions as the nuclear localization signal. These results suggest that the mechanism of the nuclear transport of EC-SOD involves a series of N-terminal signal peptide- and C-terminal heparin-binding domain-dependent processes of secretion, re-uptake and the subsequent nuclear translocation. The findings herein provide support for the view that the role of EC-SOD is to protect the genome DNA from damage by reactive oxygen species and/or the transcriptional regulation of redox-sensitive gene expression.

Keywords: Extracellular superoxide dismutase; Nuclear localization signal; Heparin binding domain; ROS; Secretion

Superoxide dismutases (SODs, EC 1.15.1.1) are metalloenzymes that catalyze the dismutation of the superoxide anion, $O_2^{\cdot-}$, to form O_2 and H_2O_2 and play a major role in the antioxidant defense system [1,2]. Three distinct isozymes have been reported in mammals, and are characterized by the differences in their cellular localization and metal ion requirements. One SOD isozyme, extracellular SOD (EC-SOD; SOD3) [3,4] is a copper- and zinc-containing secretory enzyme, which is located in extracellular fluids, including plasma [5] and the extracellular matrix of tissues [6–8]. It should be emphasized that, compared to the other two SOD isozymes, cytosolic Cu,Zn-SOD and mitochondrial Mn-SOD [9–12], the in vivo role of EC-SOD is very poorly understood.

The primary structure of murine EC-SOD indicates that the enzyme consists of an N-terminal hydrophobic signal peptide for secretion, a Cu,Zn-SOD-like domain in the middle portion, and a heparin-binding domain, which contains clusters of positively charged amino acids, in the C-terminal portion, as well as one potential site for N-linked glycosylation [13]. The heparin-binding domain provides the basis for its affinity for heparin analogues, one of the most characteristic properties of EC-SOD [14–17]. It is thought that, after being released into the extracellular space, EC-SOD is distributed to specific regions of the extracellular matrix or on the cell surface by virtue of its affinity for heparin [8,18]. This feature is believed to permit EC-SOD to efficiently scavenge superoxide in tissues.

Reactive oxygen species (ROS) such as superoxide and its derivative, H_2O_2, exert a great influence on living organisms. For example, a hydroxyl radical can

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0006-291X/02/$ - see front matter
PII: S0006-291X(02)00804-5

be generated from H_2O_2 through the Fenton reaction in the presence of transition metals, such as Cu and Fe, and superoxide as an electron donor, and this radical damages DNA [19] as well as other biologically important molecules. Trace levels of ROS are also thought to promote the transcription of several genes through the activation of transcription factors such as AP-1 or NF-κB [20,21]. However, the issue of whether ROS-scavenging systems, including SODs, participate in the regulation of transcription, has not been clarified.

In a previous study, we reported on an immunohistochemical study of EC-SOD in which an intense staining of connective tissues and vascular walls in several mouse tissues was clearly observed [18,22]. In addition, we also detected the distinct nuclear staining of EC-SOD in the cells of most of the tissues examined. In this study, we demonstrate the nuclear localization of EC-SOD in vivo and in vitro, and report on an investigation of the mechanism of the nuclear translocation of EC-SOD using several EC-SOD mutants. The findings suggest that the heparin-binding domain of EC-SOD serves dual functions, i.e., as a nuclear localization signal (NLS) and as the domain which confers the affinity for heparin analogues.

Materials and methods

Immunohistochemistry. Air-dried frozen tissue sections were processed for immunohistochemical analysis using affinity-purified anti-(mouse EC-SOD) rabbit IgG [18]. Immunoreactivity was detected with an HRP (horse radish peroxidase)-labeled donkey anti-rabbit IgG (Chemicon) and an H_2O_2–DAB chromophore system. Counter staining was carried out using hematoxylin. A negative control was performed for the testis section using pre-absorbed IgG [18].

Cell culture. COS-1 monkey cells were maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS). CHO-K1 cells were maintained in D-MEM/F-12(Ham) 1:1 medium (Lifetechnologies) supplemented with 5% FCS. 3T3-L1 mouse fibroblast cells were maintained in D-MEM medium supplemented with 10% FCS.

Transient and stable transfection. For the transient expression of mutant EC-SODs, 2 μg of expression plasmids (pcDLSRα286-derived clones) were transfected into COS-1 cells using the FuGENE6 reagent (Roche Diagnostics) following the manufacturer's recommendations. For establishing the stable overexpression in EC-SOD cell lines, pcDNA3.1(−) (Invitrogen) derived expression vectors were transfected into CHO-K1 cells, using the LipofectAmine2000 reagent (Invitrogen) following the manufacturer's instructions. For the selection of positive clones, Geneticine (G-418, Invitrogen) were supplemented with 0.8 mg/ml in D-MEM/F-12 medium. Screening for overexpression was carried out by immunocytochemical staining and Western blot analysis using anti-(mouse)-EC-SOD antibody as described below.

Preparation of nuclear extract from tissues. Approximately 2 g of mouse tissue was collected and the nuclei were prepared by centrifugation using a discontinuous gradient of OptiPrep (Nycomed Pharma, Oslo, Norway). The obtained nuclei were homogenized in 0.5 ml of 10 mM pipes buffer (pH 6.8) containing 300 mM sucrose, 3 mM $MgCl_2$, 25 mM NaCl, 1 mM EGTA, and 0.5% (w/v) Triton X-100 with a Dounce homogenizer and incubated with DNase I (700 U/ml) on ice for 2 h. The protein concentration was determined by means of a BCA protein assay kit (Pierce) with bovine serum albumin (BSA) as a standard.

Preparation of cytosolic and nuclear fractions from cultured cells. Cells were dispersed by treatment with trypsin/EDTA, harvested in phosphate-buffered saline (PBS) containing 2% FCS, and then centrifuged at 300*g* for 5 min, followed by two washings with ice-cold PBS. The cell pellet obtained was re-suspended in the hypotonic buffer [10 mM Tris–HCl (pH 8.0), 0.5 mM EDTA, 2 mM $MgCl_2$, and 2 mM 2-mercaptoethanol] with 0.3% (w/v) NP-40 and incubated on ice for 10 min to allow for swelling, then homogenized by a gentle stroke in a loosely fitted Dounce homogenizer. After centrifugation at 400*g* for 10 min, the supernatant was saved as a cytosolic fraction and the nuclear pellet was washed twice in hypotonic buffer. The expression of mutant EC-SOD in cell extracts (5 μg/lane) and cultured media (15 μl/lane) was analyzed by Western blotting as described below.

Immunocytochemical detection. A small portion of the transfected cells was diluted with the medium and transferred onto a glass slide, and then cultured for an additional 12 h. The cultures were fixed with 3.7% formaldehyde/PBS (pH 7.3) for 10 min at room temperature, permeablized with 0.1% Triton X-100/PBS for 10 min, and blocked with 2% (v/v) FCS/PBS. For indirect immunofluorescence analysis, cells were incubated with the specific antibodies anti-(mouse EC-SOD) IgG [18] in 2% FCS/PBS for 1 h at RT. The immunoreactivity was visualized using an FITC-conjugated anti-(rabbit or goat IgG) IgG (DAKO). Three washes with 2% FCS/PBS were carried out between each step.

Western blot analysis. SDS–PAGE and electroblotting procedures have been described elsewhere [22]. Samples were fractionated through 12.5% SDS–PAGE and electroblotted onto a ProBlot PVDF membrane (Applied Biosystems). For immunological detection, after treatment with a blocking solution (25 mM Tris–HCl, pH 7.5, containing 500 mM NaCl and 3% (w/v) BSA (bovine serum albumin), the blotted membrane was incubated with 1 μg/ml of the affinity-purified anti-(mouse EC-SOD) IgG in the blocking solution at RT for 2 h. The bound IgG was detected by chemiluminescence using an alkaline phosphatase-conjugated anti-(rabbit IgG) IgG (DAKO, Glostrup, Denmark) and CDP-Star (Roche) followed by exposure to XAR film (Kodak).

Construction of mutant EC-SOD expression plasmids. Mutant EC-SOD genes were generated by PCR, and oligonucleotide-mediated site directed PCR mutagenesis were carried out using the wild type EC-SOD cDNA clone as a template (pBS-MEC1212: isolated from mouse kidney cDNA library; T. Ookawara et al., unpublished data). The combinations of primers for each mutant were as follows: EK: ECF (cggaattCAGAGAACCTCAGCCATGTTG) and KPR (ttggtaccGA GGCTTAAGTGGTCTTGCA); SK: ESF (cggaattcTGGCTCTGTC ACCATGTCAAA) and KPR. To generate the 3′-truncated construct (ESK, and SSK), the first PCR was performed using the 5′-primer and STP (TTCTTGCGCTACTTTGTCTG; substituted GAG (Glu^{214}) to TAG (AMB)), and a second PCR was then carried out adding KPR primer. The PCR fragments were digested at both ends with *Eco*R I and *Kpn* I and cloned into pBluescriptII KS+ phagemid (Stratagene). For a point mutation in the heparin-binding domain (RG218: CCGCCCCTTCTTGCGCTCCTT; substituted CGG (Arg^{218}) to GGG (Gly)), PCR and oligonucleotide-mediated site directed PCR mutagenesis was carried out using an SK clone as a template. The first PCR was performed by EC213 (GAGAAGATAGGCGACACG CA) and the mutation primer (218RG), and the second PCR was then performed by adding the KPR primer. The resulting PCR fragments were digested at both ends with *Bgl*II and *Afl*II, and then inserted into the *Bgl*II and *Afl*II sites of the EK (EK-RG218) or SK (SK-RG218) clone. For the generation of stable expression cell lines, some of the constructs were re-inserted into the *Eco*RI and *Kpn*I sites of pcDNA3.1(−) mammalian expression vector (invitorogen).

Results

Nuclear-localization of EC-SOD in vivo

As shown in Fig. 1, immunohistochemical analysis of mouse tissues clearly revealed the distinctive nuclear localization of EC-SOD in thymus and testis cells. Appreciable levels of nuclear EC-SOD positive lymphocytes were scattered in the thymus tissue (Fig. 1A). The distribution of nuclear EC-SOD-positive cells in the testis was extremely characteristic of the deep nuclear staining on spermatogonia and the extent of nuclear EC-SOD accumulation was different among the sections obtained from seminiferous tubules (Fig. 1B). On the other hand, no positive signals were detected when the section was stained using the antibody which had been pre-absorbed with the purified protein (Fig. 1C), and this absence of signals verified the specificity of the antibody. Furthermore, the nuclear localization of EC-SOD was confirmed by Western blot analysis of nuclear extracts from the testis and thymus, as shown in Fig. 1D. These results show that EC-SOD can be localized in the nucleus, thus suggesting that EC-SOD potentially exists in vivo in two forms, namely a secretory form and a nuclear form.

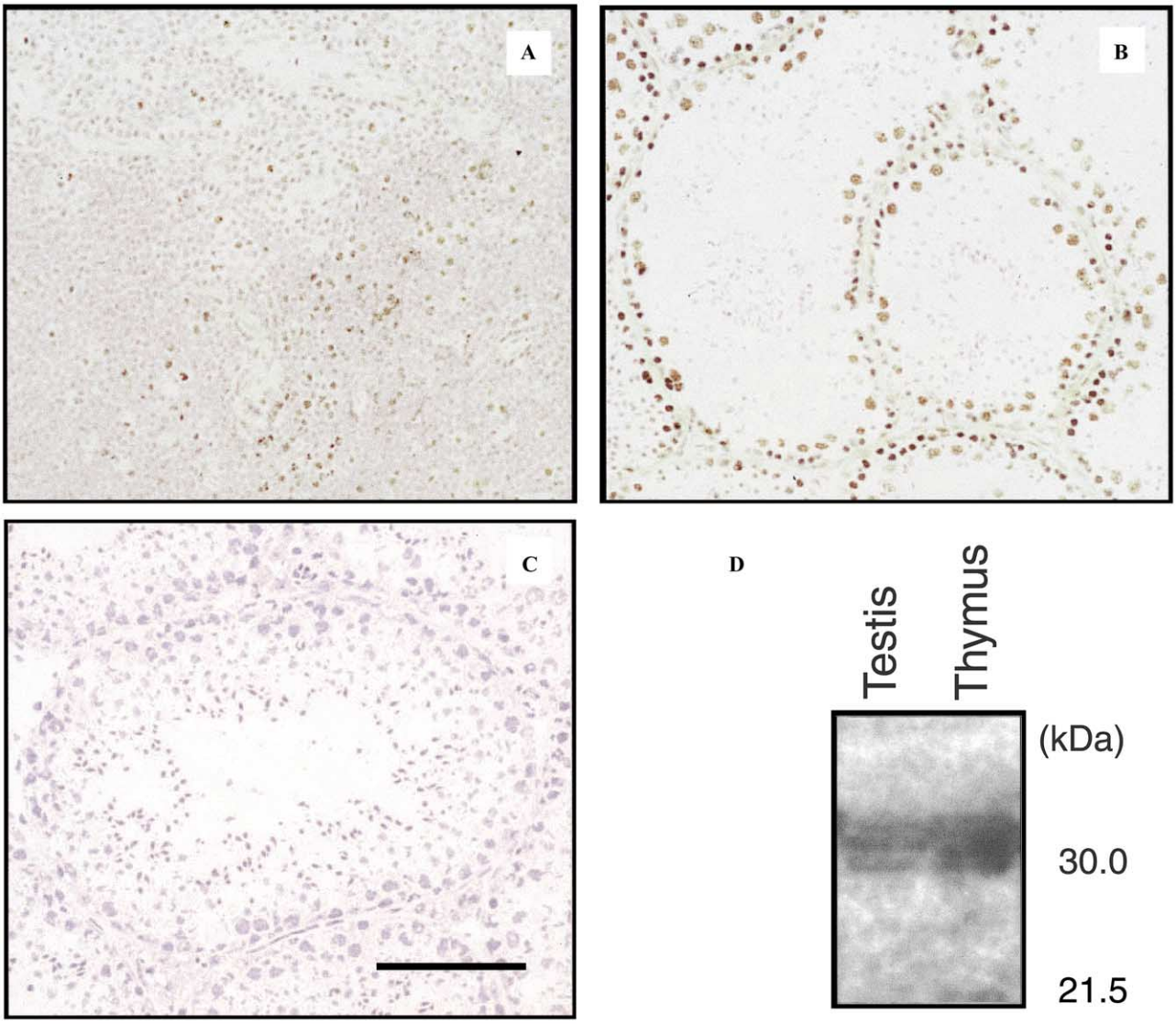

Fig. 1. Nuclear localization of EC-SOD in vivo. Air-dried frozen tissue sections from mouse thymus (A) and testis (B) were processed for immunohistochemical analysis using affinity-purified anti-(mouse EC-SOD) rabbit IgG (detailed in [18]). Immunoreactivity was detected by an H_2O_2–DAB choromophore system and counter staining was carried out using hematoxylin. The bar indicates 100 μm. A negative control was performed for the testis section using pre-absorbed IgG ([20]; C). Western blot analysis of nuclear extracts from testis and thymus were also performed to detect EC-SOD in the nucleus (D).

Subcellular localization of EC-SOD mutants and the involvement of the heparin-binding domain in nuclear translocation

The cDNA sequence indicates that the wild type EC-SOD contains a hydrophobic signal peptide at the N-terminus, the second AUG codon at the end of the signal peptide, and a positively charged heparin-binding domain near the C-terminus, which consists of four arginine and three lysine residues in the sequence of eight consecutive amino acids (–K–E–R–K–K–R–R–R–; residues 213–220 [13]) (Fig. 2A). This domain is similar to the nuclear localization signal (NLS) of other nuclear proteins [23,24]. Therefore, to examine the actual role of this domain in translocation to the nucleus, we prepared several EC-SOD mutants which lacked either or both of the signal peptides and this putative NLS by a truncation (Fig. 2B, 2C) or an amino acid substitution (Fig. 2C), and transiently expressed these mutants in COS cells. The distribution of the wild type and mutant EC-SODs in the transfected COS cells was analyzed by immunofluorescence microscopy (Fig. 3). While the wild type (EK) showed evidence of cytoplasmic staining, the SK mutants, which lack the signal peptide, display distinct nuclear staining (Fig. 3A), suggesting that EC-SOD contains a possible functional

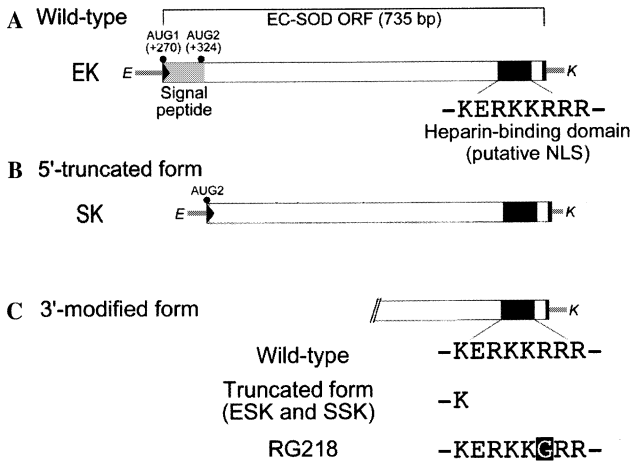

Fig. 2. Schematic representation of the mutant EC-SOD cDNA inserts in the expression plasmids. (A) The wild type EC-SOD (EK) contains a complete open reading frame (ORF) of 756 bp in length and the recombinant protein should be a wild type. Black dots represent positions of AUG codons for translation initiation. A signal peptide, the putative NLS (heparin-binding domain), the position of translation initiation, and the stop codon are depicted by a shadowed box, a black box, a sidewise black triangle, and a black line, respectively. Restriction endonuclease sites are shown for *Eco*RI (E) and *Kpn*I (K). (B) Gene construct of the 5′-truncated forms. SK has an ORF, from which the signal peptide sequence including AUG1 was deleted, and the translation would be started from AUG2. The recombinant protein of SK should be a non-secretory form. (C) Truncation or amino acid substitution at the putative NLS sequence was achieved by one base substitution of GAG (Glu^{238}) to TAG (AMB) (ESK and SSK) or CGG (Arg^{218}) to GGG (Gly) (RG218), respectively.

domain as the NLS even though the presence of the signal peptide appeared to mask the nuclear translocation to a significant extent.

To identify the possible NLS, we investigated the issue of whether nuclear translocation is conferred by the heparin-binding domain in the C-terminal region. When subcellular localization of the C-terminus-truncated mutants was found that the loss of the domain leads to an impairment in nuclear translocation (Fig. 3B, SSK), suggesting that the heparin-binding domain plays a role in the nuclear translocation of EC-SOD.

To further confirm this, we explored the effect of the amino acid substitution in the C-terminal region of the non-secretory form (SK mutant) on the subcellular localization of the protein. A mutant (SK-RG218) in which Arg^{218} in the heparin-binding domain was replaced by Gly was prepared. As shown by immunofluorescence microscopic analysis (Fig. 3B), the SK-RG218 mutant exhibited diffuse cytoplasmic fluorescence signals, indicating that the amino acid substitution in the heparin-binding domain abolished the nuclear translocation of the enzyme. These results clearly indicate that the heparin-binding domain of EC-SOD serves as the potentially functional NLS, and it is most likely that this "NLS" is primarily responsible for the nuclear translocation of EC-SOD in the absence of the "masking" effect of the signal peptide.

Cellular uptake and nuclear translocation of wild type EC-SOD

To demonstrate the nuclear translocation of the "wild type" EC-SOD in vitro using cultured cells, we established cell lines which stably express the wild type (CHO/EK) and mutant EC-SOD (CHO/EK-RG218).

Using the EC-SOD-rich conditioned media from these stably expressed cell lines, we examined the cellular uptake of the secreted EC-SOD and its subsequent nuclear transport in 3T3-L1 preadipocytes. The conditioned medium of non-transfected CHO-K1 was used as a control. After incubation for 4 h, the cells were subjected to immunofluorescence microscopic analysis. As shown in Fig. 4A, the wild type EC-SOD was incorporated into 3T3-L1 cells from the conditioned medium whereas the RG218 mutant was not taken up by the cells. Consistent with these results, Western blot analysis also revealed that the cells took up the wild type but not the mutant (Fig. 4B, upper panel). Furthermore, the protein was detected in the nuclear fraction only when the wild type was exogenously provided (Fig. 4B, middle panel). A comparison of the molecular sizes of the wild type (CHO/EK) between cytosolic fraction and conditioned medium showed that the secreted form of the protein is larger than the cytosolic species, probably due to post translational modification, such as N-glycosylation

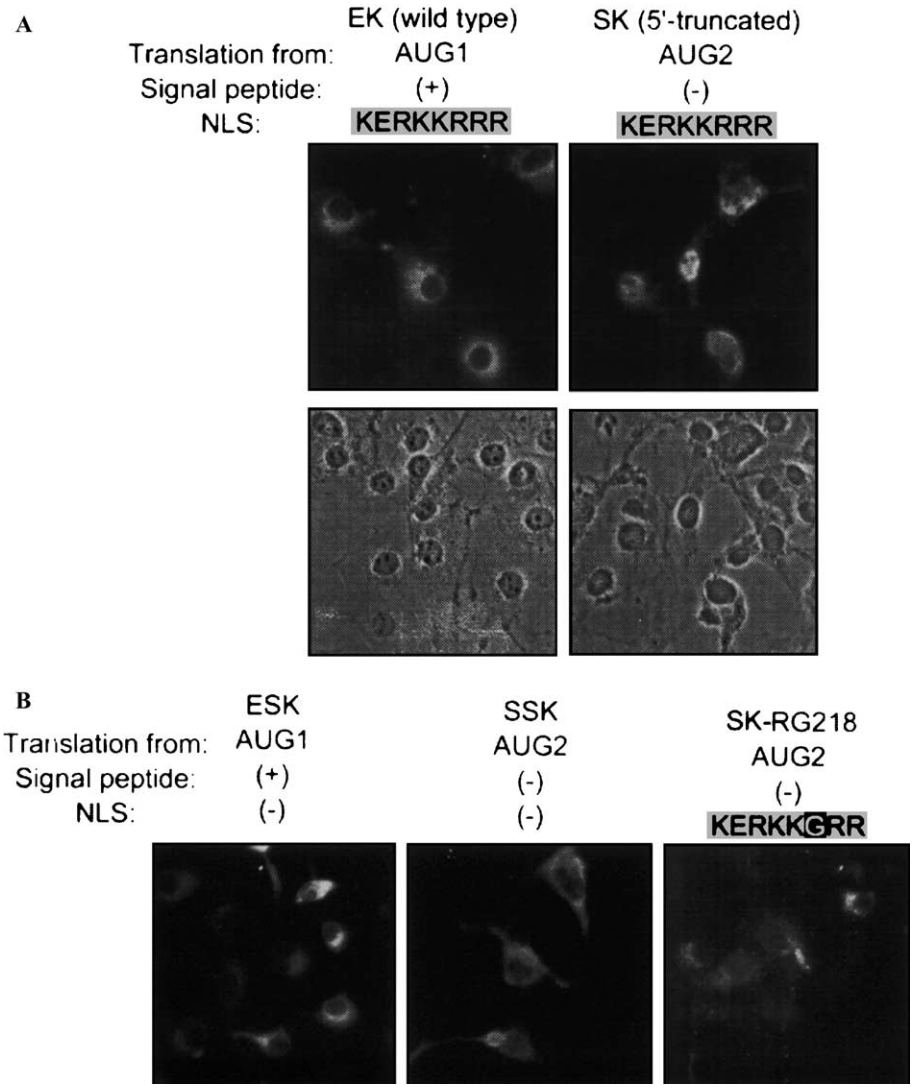

Fig. 3. Subcellular localization of wild type and mutant EC-SODs in COS-1 cells. Expression plasmids for the wild type and 5′-truncated gene constructs were transfected into COS-1 cells and the subcellular localization of the mutant EC-SODs was analyzed by an indirect immunofluorescence method using the FITC-conjugated secondary antibody. (A) Upper panels illustrate immunostaining for EC-SOD. Lower panels show phase contrast images. (B) Effects of truncation or point mutation of the heparin-binding domain (putative NLS) on nuclear targeting of mutant EC-SOD. ESK and SSK are the 3′-truncated forms of EK (wild type) and SK (5′-truncated), respectively. SK-RG218 has a single amino acid substitution in the heparin-binding domain (putative NLS) of the 5′-truncated form (SK). The expression plasmids were transfected into COS-1 cells and the subcellular localization of recombinant protein was analyzed by indirect immunofluorescence. The substituted amino acid is indicated by a white letter.

(Fig. 4C). The molecular size of the protein transported into the nucleus was found to be the same as that of the protein species in the conditioned medium (Fig. 4B, arrow b). These results suggest that EC-SOD is capable of being translocated from the extracellular space into cytosol and even the nucleus. The mechanism for the nuclear localization of EC-SOD appears to involve the secretion of the protein from the cells followed by cellular uptake and nuclear translocation. While the N-terminal signal peptide is required for secretion, the C-terminal heparin-binding domain which may also be considered to be an NLS would be expected to play essential roles in the subsequent processes in the mechanism.

Discussion

EC-SOD was originally described as a secretory isoenzyme of SOD, and it is known that the enzyme is located in extracellular fluids and connective tissues. The heparin-binding domain of EC-SOD confers affinity for heparin analogues thus allowing the enzyme to interact with proteoglycans. In the present study, we have shown that EC-SOD is localized in vivo and in vitro in the nucleus as well as in the extracellular space, and also propose a possible mechanism for its nuclear translocation.

These different localizations can be explained by a mechanism which regulates the removal of the signal

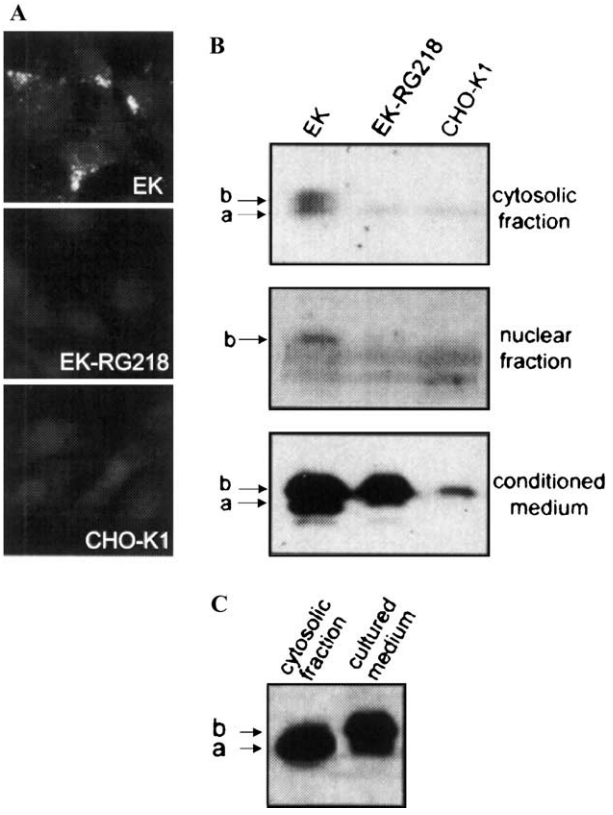

Fig. 4. Uptake and nuclear translocation of EC-SOD in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were incubated in the conditioned media of CHO/EK, CHO/EK-RG218, and non-transfected CHO-K1, and the uptake and subcellular distribution of EC-SOD in 3T3-L1 cells were then investigated by immunofluorescence microscopy (A), and Western blotting (B). Moleclular sizes were compared between cytosolic and secreted EC-SOD from CHO/EK (C). Arrows a and b, indicate the sizes of cytosolic EC-SOD, and nuclear EC-SOD, respectively (B and C).

peptide, since a loss of this peptide evidently converts the enzyme into a nucleus-localizing protein as long as the heparin-binding domain is intact (Fig. 3). A genomic Southern blot analysis indicated the presence of a single copy of the EC-SOD gene in mice, and, in addition, an analysis of transcripts such as 5′-RACE did not show the presence of the transcript which encodes a signal peptide-minus EC-SOD as the result of alternative initiation or processing (data not shown). Therefore, it is unlikely that the elimination of the signal peptide is executed at the nucleotide levels.

Since, in general, the signal peptide-dependent targeting of the secretory proteins to the ER is a co-translational process, it would be reasonable to conclude that the N-terminal signal peptide which directs the proteins to the ER/Golgi system is dominant to the C-terminal putative NLS. If EC-SOD were to undergo cleavage of the signal peptide without targeting to the ER and was, thereafter, translocated to the nucleus, the apparent molecular mass of the protein should have been reduced due to the loss of the signal peptide and the absence of N-glycosylation. In this case, the molecular size would be expected to be significantly smaller, compared to the secreted form of the enzyme. However, the size of >30 kDa in the tissues was larger than that of non-glycosylated form (Fig. 1), and it therefore seems more likely that EC-SOD which was detected in the nuclear fraction is glycosylated. In vitro studies using transfected cells also suggest that the origin of this nuclear EC-SOD is via the uptake of secreted EC-SOD but not a direct translocation of the cytosolic EC-SOD. These findings and considerations led us to conclude that EC-SOD is localized in the nucleus via a sequence involving secretion, re-uptake, and subsequent nuclear translocation.

Since the ''re-uptake-nuclear translocation'' mechanism which we propose for EC-SOD is based on findings obtained in in vitro studies using cultured cells, it cannot definitely be concluded that the EC-SOD is localized in the nuclei in all tissues where the nuclear localization of the enzyme was observed in immunohistochemical studies. Considering the recent reports on some growth factors which contain both the secretory signal peptide and NLS, such as fibroblast growth factors (FGFs) [25–27], it is possible that, in some tissues, EC-SOD might be translocated to the nucleus in a manner independent of the mechanism proposed herein. However, although such an alternative mechanism for the nuclear translocation of EC-SOD is not necessarily excluded, it seems certain that the re-uptake-nuclear translocation mechanism is, at least, one of the mechanisms for allowing the enzyme to be transferred to the nucleus, as is clearly suggested in our present study. In fact, it has been reported that exogenously added FGF-1 is incorporated into cells and is translocated to nuclei more efficiently than endogenous FGF-1 [28]. This unique mechanism could be common in some proteins which are capable of behaving as both secretory and nuclear forms, such as EC-SOD and FGF.

At present, the mechanism for EC-SOD uptake is not clear. However, the interaction of the heparin-binding domain of EC-SOD with cell surface proteoglycans may be a critical factor in the cellular uptake of the enzyme, as shown by the finding that a single amino acid substitution in the heparin-binding domain (EK-RG218 mutant) led to the complete inhibition of cellular uptake. The efficiency of the uptake was much different among the cell lines examined in this study. 3T3-L1 cells had a very high efficiency, CHO-K1 cells were moderate, and COS cells were very low (data not shown). Compositions of the cell surface proteoglycans may play an important role in the initial association of the enzyme with the cells, and thereby determine the efficiency of the uptake at this step. This could account for the tissue- or cell type-specificity of the nuclear accumulation of EC-SOD, as found in the immunohistochemical examination.

Three SOD isozymes are known in mammals, and their localizations are the cytoplasm for Cu,Zn-SOD, the mitochondria for Mn-SOD, and the extracellular space for EC-SOD. However, a "nuclear form" of SOD has not been fully investigated in spite of the obvious importance of protecting genomic DNA from ROS, but EC-SOD appears to serve as such a nuclear isozyme. Therefore, one possible role of this isozyme would be to serve as a scavenging factor of $O_2^{\cdot-}$ in the nucleus. Since it is known that DNA is cleaved via attack by ROS [19], genome DNA can potentially be damaged as the result of the accumulations of $O_2^{\cdot-}$. It is probable that ROS, including $O_2^{\cdot-}$, can interact with Cu ions bound to DNA, ultimately forming ·OH via reactions known as Fenton chemistry or the Habor–Weiss reaction. Indeed, Cu ion is an abundant and important component metal ion in chromatin, and potentially could be a factor in DNA base damage [29]. Thus, EC-SOD may be involved in the protection of genomic DNA.

On the other hand, it is also possible that the nuclear type EC-SOD modulates signal transduction as a scavenger of $O_2^{\cdot-}$ or a supplier of H_2O_2. Oury et al. [4] suggested that EC-SOD could serve as a modulator of the nitric oxide signal, especially in the extracellular space. It has been suggested that, in the nucleus, trace levels of ROS such as $O_2^{\cdot-}$ and H_2O_2 activate transcription through the antioxidant responsive element [30,31] and/or other elements [32]. In fact, it is well known that the activities of several transcription factors including p53, NF-κB, and AP-1 [33,34] are regulated by the redox state at both the transcriptional and posttranslational levels.

In terms of the damage of genomic DNA by ROS and the involvement of ROS in the regulation of gene expression, it seems physiologically reasonable that an SOD isozyme is also distributed in the nucleus. The findings in this study provide a new insight into the ROS-scavenging system which may contribute to genome stability or the control of gene expression, although the complete understanding of the role of the nuclear EC-SOD will require further investigations.

Acknowledgments

We thank Dr. Yoshihiro Yoneda for helpful discussions and information, Dr. Tetsuji Nakai and Dr. Tetsuo Sato for providing us with mouse tissues, and Masahiko Segawa for skillful technical assistance.

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