Molecular Cloning and Functional Analysis of Apoxin I, a Snake Venom-Derived Apoptosis-Inducing Factor with L-Amino Acid Oxidase Activity†

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ABSTRACT: We previously purified apoxin I, an apoptosis-inducing factor with L-amino acid oxidase (LAO) activity, from Western diamondback rattlesnake venom. To determine the primary structure of apoxin I, we cloned its cDNA. The amino acid sequence showed that apoxin I has an FAD binding domain and shares homology with L-amino acid oxidase (LAO) from *Neurospora crassa*, human monoamine oxidase B, and mouse interleukin 4-induced F1G1 protein. The full-length apoxin I has an N-terminal signal sequence that is processed in mature apoxin I in venom. When the apoxin I gene was transfected into human 293T cells, the recombinant protein was expressed in the cells, and a significant amount of apoxin I was secreted into the medium. The secreted recombinant apoxin I protein showed LAO and apoptosis-inducing activity, but the recombinant protein in the cells did not, suggesting that maturation and secretion of the apoxin I protein is needed for its activity. Treating the transfected cells with tunicamycin inhibited the secretion and LAO activity of the recombinant apoxin I. In addition, deleting the amino-terminal region flanking the signal sequence, the FAD-binding domain and the carboxy-terminal region abolished the secretion and LAO activity of the recombinant proteins. These results indicate that in order for apoxin I to become active, these regions and posttranslational modification, such as N-glycosylation, are required.

Venom of the Western diamondback rattlesnake (*Crotalus atrox*) induces apoptosis in human umbilical vein endothelial cells (*1*), which could result in hemorrhage in the area of the snake bite. In previous studies to identify the hemorrhagic factor, we purified an apoptosis-inducing factor, apoxin I, from rattlesnake venom (*2*). The amino-terminal sequences of the purified apoxin I was very similar to L-amino acid oxidases (LAO)[1] of the Malayan pit viper and the king cobra (*3*), and the purified apoxin I actually oxidized L-leucine, but not D-leucine, to produce H_2O_2. Studies with H_2O_2 scavengers showed that the H_2O_2 produced upon L-amino acid oxidation by apoxin I plays a major role in inducing apoptosis (*2*).

To identify the primary structure of apoxin I, we cloned apoxin I cDNA. Apoxin I has a consensus motif to bind FAD and shares homology with LAO from *Neurospora crassa*, human monoamine oxidase B, and mouse interleukin 4-induced F1G1 protein. We expressed the recombinant apoxin I in eukaryote cells and found that enzymatically active protein was secreted into the medium. In the cells, however, activity of the recombinant protein is suppressed. The biogenesis of apoxin I is discussed herein.

EXPERIMENTAL PROCEDURE

Purification, Peptide Fragments, and Amino Acid Sequence. Lyophilized crude venom from the Western diamondback rattlesnake was purchased from Sigma, and apoxin I was purified to a homogeneous single band, as described previously (*2*). The purified apoxin I was digested with lysyl endoproteinase from Lysobacter enzymogenes (Boeringer mannheim, Basel, Switzerland) in 50 mM Tris/HCl, pH 8.5, at 37 °C for 16 h. The peptide fragments obtained were separated by reversed-phase HPLC on a column of Puresil C18 (Waters, Milford, MA) using a linear gradient (0 to 60%) of 2-propanol/acetonitrile (7:3) in distilled water containing 0.1% TFA in 60 min at a flow rate of 1 mL/min. Elution profiles were monitored by the absorbance at 220 nm, and the peptide fragments were manually collected. The amino acid sequence analyses of the peptide fragments fractionated by HPLC were performed with a PSQ-1 gas-phase sequencer (Shimadzu, Kyoto, Japan).

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[1] Abbreviations: LAO, L-amino acid oxidase; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; NBD C_6-ceramide, 6-[(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl]sphingosine; ER, endoplasmic reticulum; ORF, open reading frame.

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A

Signal Sequence

MNVFFMFSLLFLAALGSCAHDRNPLEECFRETDYEE A1
FLEIAKNGLTATSNPKRVVIVGAGMAGLSAAYVLAG
AGHQVTVLEASERVGGRVRTYRKKDWYANLGPMRLP A2
TKHRIVREYIKKFDLKLNEFSQENENAWYFIKNIRK
RVREVKNNPGLLEYPVKPSEEGKSAAQLYVESLRKV
VKELKRTNCKYILDKYDTYSTKEYLLKEGNLSPGAV
DMIGDLLNEDSGYYVSFIESLKHDDIFGYEKRFDEI
VGGMDQLPTSMYEAIKEKVQVHFNARVIEIQQNDRE A3
ATVTYQTSANEMSSVTADYVIVCTTSRAARRIKFEP
PLPPKKAHALRSVHYRSGTKIFLTCKKKFWEDDGIR
GGKSTTDLPSRFIYYPNHNFTSGVGVIIAYGIGDDA
NFFQALDFKDCADIVINDLSLIHQLPKEDIQTFCRP
SMIQRWSLDKYAMGGITTFTPYQFQHFSEALTAPFK A4
RIYFAGEYTAQFHGWIDSTIKSGLTAARDVNRASEN
PSGIHLSNDNEF

B

A2

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APOXIN I          31  SNPKRVVIVGAGMAGLSAAYVLAGAGH-QVTVLEASERVGGRVRTYR-KK-DW-----YANLGPMRL 89
LAO(Neurospora)  173  AKSKNIAIVGAGMSGLMTYLCLTQAGMTNVSIIEGGNRLGGRVHTEY-LS-GGPFDYSYQEMGPMRF 237
MAO-B              2  SNKCDVVVVGGGISGMAAAKLLHDSGL-NVVVLEARDRVGGRTYTLRNQK-VKYVD-----LGGSYV 61
FIG1              56  SKPQKVVVVGAGVAGLVAAKMLSDAGH-KVTILEADNRIGGRIFTFRDEKTGW-----IGELGAMRM 116
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A3

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APOXIN I         226  GYEKRFDEIV-GGMDQLPTSMYEAIKEKV------QVHFNARVIEIQQNDREATVTYQTSANE-MSSVTADY-VIVCTTS  296
LAO(Neurospora)  405  TFPRRRGAID-GGLNRLPLSFHPLVDNAT------TLNRRLERVAFDAETQKVTLHSRNSYKDSFESSEHDYAVI-AAPF  476
MAO-B            193  GGTTRIISTTNGGQERKFVGGSGQVSERIMDLLGDRVKLERPVIYIDQT-REN-VLVETLNHE-M--YEAKY-VISAIPP  266
FIG1             253  SDRLRYSRIV-GGWDLLPRALLSSLSGAL------LLNAPVVSITQGRNDVRVHIATSLHS-EKTLTADVVLLTAS-GPA  319

APOXIN I         297  RAARRIKFEPPLP---KKAHALRSVH-YRSGTK-IFLTC-KKKFWE 337
LAO(Neurospora)  477  SIVKKWRFSPALDLTAPTLANAIQNLE-YTSACK--VALEFRTRFWE 520
MAO-B            267  TLGMKIHFNPPLPM---MRNQMITRVPLGSVI-KCIV-YY-KEPFWR 307
FIG1             320  LQ--RITFSPPLTR---KR-QEALRALHYVAASK-VFLSF-RRPFWH 357
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A4

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APOXIN I         419  R-WSLDKYAMGGI-TTFTPYQFQH-FSEAL--TAPFKRIYFAG-EYTA-QFHGWIDSTIKSGLTAA-RDVNRA  483
LAO(Neurospora)  608  RCWALDPLESASWAS-PTVGQHELYLPEYF-QTRNNL--VFVG-EHTS-YTHAWIASALESGIRGSVQLLLEL  674
MAO-B            386  KNWCEEQY-SGGCYTTYFPPGILTQYGRVLRQ--PVDRIYFAGTE-TATHWSGYMEGAVEAGERAA-REILHA  448
FIG1             440  R-WAEDPH-SQGGFVVQ-PPLYGRE-AEDYDWSAPFGRIYFAG-EHTA-LPHGWVETAVKSGLRAAVR-INNN  505
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FIGURE 1: (A) Amino acid sequence of apoxin I deduced from cDNA sequence. Potential FAD binding domain (A2) and other conserved regions (A1, A3, and A4) are shaded. The white box shows the N-terminal signal sequence that is processed in the purified apoxin I protein from venom. The peptide sequences determined from purified Apoxin I protein are underlined. The potential N-glycosylation site is underlined twice. (B) An alignment of the amino acid sequence of the FAD binding domain (A2) and the conserved regions (A3 and A4) is presented.

Cloning and Sequencing the Apoxin I Gene. Part of a sequenced peptide (underlined here), DWYANLGPMR-LPTSN, was converted to a reverse complimentary oligonucleotide sequence, CAT IGG ICC IAI ITT IGC ITA CCA, as a degenerate antisense primer including inosine residues. The T3-promoter sequence on the lambda-ZAP phage vector, AAT TAA CCC TCA CTA AAG GG, was used as the sense primer, and PCR was carried out using the cDNA library of a *C. atrox* venom gland (*4*) as a template DNA. The amplified DNA was sequenced, and a second PCR was performed with TGT AGG AGG ACG AGT GAG GA as the sense primer and T7 promoter sequence GTA ATA CGA CTC ACT ATA GGG C as the antisense primer. The PCR-amplified DNAs were inserted into pCRII vector (Invitrogen, CA) and sequenced by the dideoxy chain termination method using a Sequence kit RPN2438 (Amersham) and model 4000A DNA sequencer (Li-Cor). The sequence of apoxin I cDNA was confirmed by sequencing at least five independent PCR-amplified clones. The resulting amino acid sequences

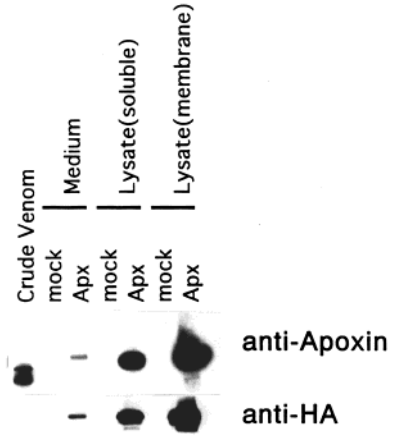

FIGURE 2: Expression of recombinant apoxin I in 293T cells. HA-tagged apoxin I was transfected into 293T cells. Conditioned medium, and soluble and membrane fractions of the cells were analyzed by western blot with anti-apoxin I (upper panel) and anti-HA (lower panel).

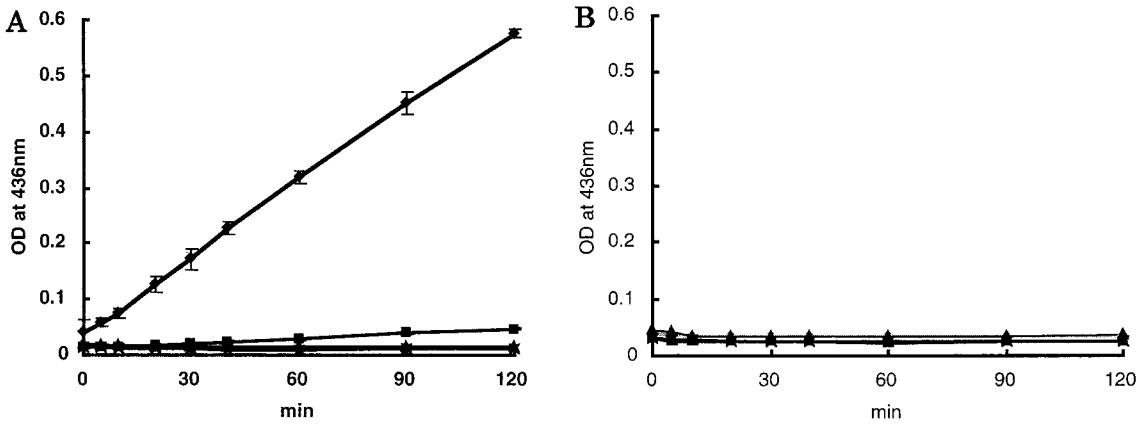

FIGURE 3: LAO activity of the recombinant apoxin I. Amino acid oxidase was measured with conditioned medium (diamond) and cell lysate (square) from apoxin I-transfected cells using L-leucine (A) and D-leucine (B) as substrates. Conditioned medium (triangle) and cell lysate (cross) from mock-transfected cells were also examined as controls.

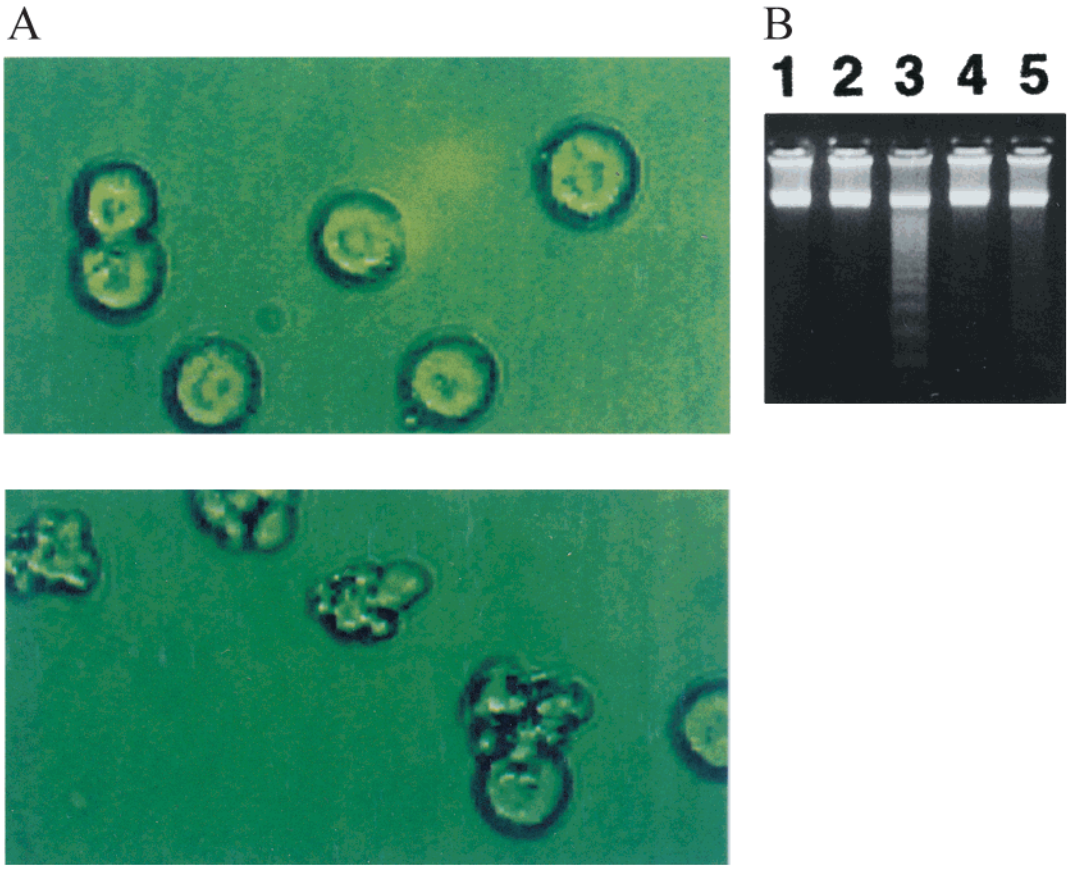

FIGURE 4: Apoptosis-inducing activity of recombinant apoxin I. The apoxin I gene was transfected into 293T cells. After 48 h, the conditioned medium was collected and added to the culture of HL-60 cells for 4 h. (A) Microscopic observation of HL-60 cells treated with the conditioned medium from mock-transfected 293T cells (upper panel) and apoxin I-transfected 293T cells (lower panel). (B) DNA fragmentation assay. HL-60 cells were treated with the conditioned medium from mock-transfected cells (lane 1), lysate from apoxin I-transfected cells (lane 2), conditioned medium from apoxin I-transfected cells in the absence (lane 3) or presence of 1000 units/mL of catalase (lane 4) and 2 mM Trolox (lane 5).

were analyzed and aligned with a related sequence using BLAST and FASTA applications.

Construction of Expression Vectors. The full-length cDNA of apoxin I ORF was inserted into *Xba*I and *Pst*I restriction enzyme site of pCG mammalian expression vector, which has an HA tag at the C-terminus. The *Eco*RI−*Hind*III fragment of pCG-apoxin I was subcloned into the *Eco*RI−*Hind*III site of pUC19 to construct pK900. Primers (Biologica) to generate deleted genes of apoxin I were as follows: AX-2 primer, 5′-AAG CTT CAT GGA TGT AGG CAA CTG ATC CAT TCC ACC-3′; AX-3 primer, 5′-GGA TCC TGA GAT TGC CAA AAA TGG TCT GAC AGC GAC-3′; AX-4 primer, 5′-GGA TCC TGG TGC AGG AAT GGC TGG GCT TAG TGC AGC CT-3′; AX-7 primer, 5′-AAG CTT GGA TCC TGT GCA CAG CTT CCC AAG GCA GCC AAG AAC AGC-3′; M13Frd primer, 5′-TGT AAA ACG ACG GCC AGT-3′; AX-9 primer, 5′-GGA TCC AGA AAT TCT TCA TAG TCA GTT TCT CGG-3′; AX-

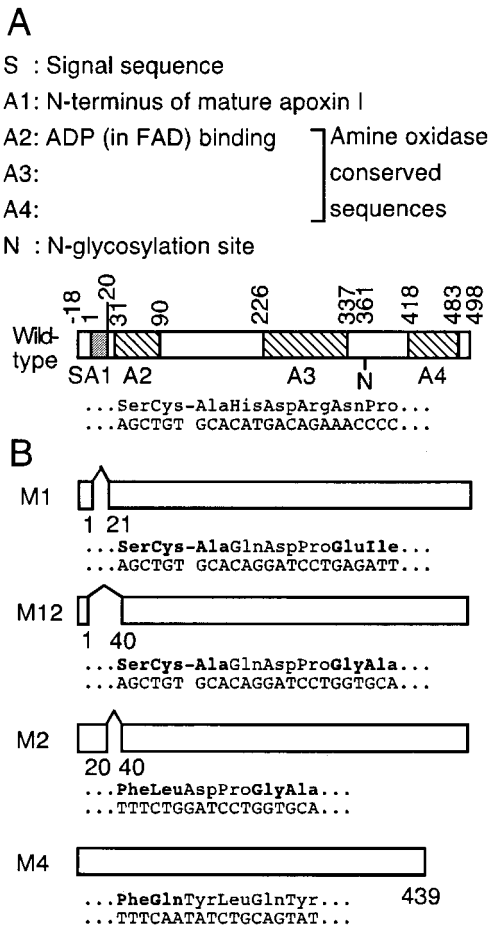

FIGURE 5: Schematic diagram of the structure of apoxin I and its deletion mutants. (A) Structure of apoxin I. Numbers on the box indicate amino acid residue numbers. (B) Structure of deletion mutants. Terminal residue numbers remaining in deletion mutants are shown. The junction sites of the deletions were indicated with the original amino acid sequence of apoxin I in bold.

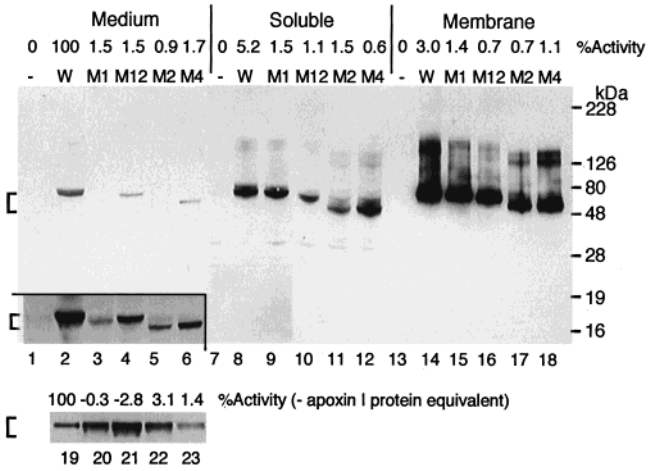

FIGURE 6: Western blot analysis and LAO activity of deletion mutants. The 293T cells were transfected with wild (W) and deletion mutants (M1−M4) of apoxin I. The conditioned media (lanes 1−6), cellular soluble (lanes 7−12), and membrane (lanes 13−18) fractions were prepared, and 10 μL of each fraction was subjected to western blot analysis and LAO assay. The LAO activity in the medium fraction of wild apoxin I (lane 2) is shown as 100%, and the percentage of activity in each fraction is presented. (−) Mock transfected without DNA. The inset shows longer exposure of the medium fractions. The recombinant apoxin I protein in the medium fraction was adjusted, and LAO activity was measured and further normalized by the intensity of the protein bands (lanes 19−23, % activity). In some cases, the calculation resulted in slight negative numbers showing no activity.

11 primer, 5′-TTT GAA CCA CCC CTT CCG CCA AAG AAA GC-3′; AX-12 primer, 5′-CTG CAG ATA TTG AAA CTG GTA GGG AGT GAA GGT GG-3′. To introduce the *Bam*HI site after the signal sequence (Figure 1, M1 and M12, GGATCC in the DNA sequences), gene coding for the region was amplified by PCR using primers AX-7 and M13Frd, with pK900 as a template. The *Eco*RV−*Ecl*I fragment of the amplified fragment was cloned into the *Ecl*I site of pK900 to construct pK909. The gene coding for the M1 mutated region was amplified by PCR using primers AX-2 and AX-3, with pK900 as a template. The amplified fragment was cloned into pK909 and into pCGBL-apoxin I to construct the M1 gene. The same procedure was followed using primers AX-2 and AX-4 to construct the M12 gene (pK919). The PCR was performed using primers M13Frd and AX-9 with pK900 as a template. The *Xba*I−*Bam*HI fragment of the amplified portion was cloned into pK919 to construct M2. A PCR was performed using primers AX-11 and AX-12 with pK900. The *Spe*I−*Pst*I fragment of amplified gene was cloned into pK900 to construct pK938. The *Xba*I−*Pst*I fragment of pK938 was cloned into the *Xba*I−*Pst*I fragment of pK919 to construct M4. All amplified sequences were confirmed by DNA sequencing.

Cell Culture and Transfection. Human promyelocytic leukemia HL-60 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and maintained in RPMI 1640 medium (Nissui) supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/mL of kanamycin in a humidified atmosphere of 5% CO_2 and 95% air. Human embryonic kidney 293T cells and NIH3T3 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/mL of kanamycin. The wild and mutated apoxin I genes were transfected into 293T and NIH3T3 cells using the calcium phosphate method (*5*). After transfection, the conditioned media and the transfected cells were individually harvested. The cells were lysed by sonication, and the lysates were centrifuged for 10 min at 15 000 rpm to prepare membrane and soluble fractions.

Preparation of anti-Apoxin I and Histochemical Study. New Zealand White rabbits were immunized by multiple subcutaneous injections of 100 μg/mL of purified apoxin I emulsified with an equal volume of Freund's complete adjuvant (Waco Pure Chemicals) for the first immunization and with Freund's incomplete adjuvant for the following immunizations. Injections were repeated biweekly, and serum was obtained after the fifth injection. To perform the immunofluorescence method, cells were grown on a chamber slide glass, fixed with 100% methanol at −20 °C, washed with PBS three times and incubated with 2 μL of anti-apoxin I antibody, 5 mg of skim milk, 198 μL of PBS for 20 min at 37 °C. The slide glass was washed three times with PBS and incubated with 2 μL of anti-rabbit IgG antibody conjugated with rhodamine (ICN), 198 μL of PBS for 20 min at 37 °C, and washed with PBS three times. The slide glass was photographed through the microscope with the proper excitation wavelength (500−550 nm). The magnification was 600-fold in all experiments.

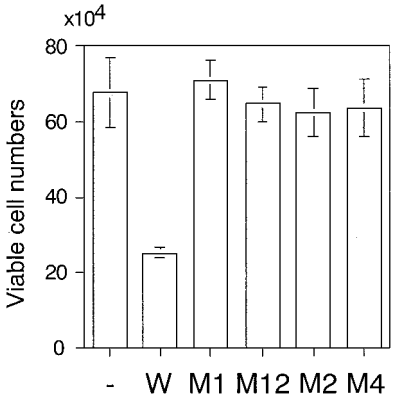

FIGURE 7: Wild apoxin I is toxic to 293T cells. The 293T cells were transfected with wild (W) and mutant (M1−M4) apoxin I genes and incubated for 24 h in 1 mL of medium supplemented with 10% serum. Fresh medium (0.5 mL) was then added, and cells were incubated further for 24 h. Viable cells were counted using trypan blue. (−) Mock transfected without DNA.

To detect endoplasmic reticulum (ER), the fixed NIH3T3 cells were washed with 10 mM Tris-HCl, pH 7.7, incubated with 100 μL of 1 μg/mL 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] (Molecular probes) (*6*) for 15 s at room temperature, and washed with 10 mM Tris-HCl, pH 7.7. To detect Golgi apparatus, fixed cells were washed with PBS, incubated with 100 μL of 1 μg/mL 6-[(*N*−(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl]sphingosine (NBD C_6-ceramide) (Molecular probes) (*7*), 10 μg/mL bovine serum albumin, for 3 min at room temperature, and washed with PBS. These preparations were directly stained using the immunofluorescence method, as described above. Excitation wavelength used in both preparations was 460−480 nm. Adobe Photoshop 4.0 was used to overlap the two different views of the same region.

Assays. Measurement of LAO activity (*3*), DNA fragmentation assay (*8*), and morphological observation of apoptosis was carried out as described previously (*2*).

RESULTS

Molecular Cloning of Full-Length Apoxin I cDNA. We previously purified the apoptosis-inducing factor, apoxin I, from Western diamondback rattlesnake venom (*2*). The purified apoxin I was digested by lysyl endopeptidase, and the resulting five fragments were analyzed for their amino acid sequences (Figure 1A, underline). According to the amino acid sequence, we designed a degenerate PCR primer and successfully amplified a fragment from the cDNA library of a *C. atrox* venom gland, and then full-length apoxin I cDNA with 1551-bp ORF was cloned.

Figure 1A shows the deduced amino acid sequence of apoxin I. The sequence was very similar to *Crotalus adamanteus* LAO, which was reported recently (*9*). The full-length apoxin I has a signal sequence at the amino terminus that is processed in mature protein purified from venom. Following the signal sequence is the amino terminus of mature apoxin I, which is homologous to various LAO from snake venoms (A1) (*3*). In addition, apoxin I has a predicted FAD binding domain (A2) (*9*−*11*) and conserved regions (A3 and A4) homologous to LAO from *Neurospora crassa* (*12*), human MAO−B (*13*), and mouse interleukin 4-induced F1G1 protein (*14*) (Figure 1B).

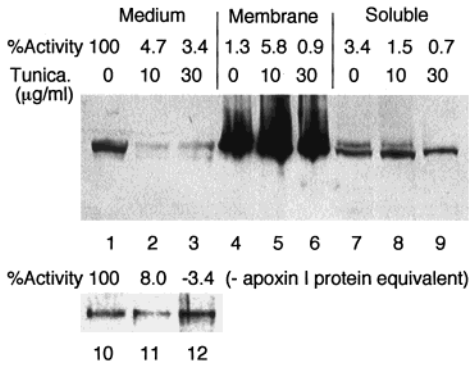

FIGURE 8: Tunicamycin inhibits production of active apoxin I. The transfected 293T cells were incubated for 48 h in the presence or absence of tunicamycin (Tunica). The conditioned medium and cell fractions were analyzed as described in the legend for Figure 6. The recombinant apoxin I protein in the medium fraction was adjusted, and LAO activity was measured and further normalized by the intensity of the protein bands (lanes 10−21, % activity). The calculation resulted in a slight negative number showing no activity.

LAO and Apoptosis-Inducing Activity of Recombinant Apoxin I. To test whether the cloned cDNA of apoxin I encodes a functional apoptosis-inducing protein with LAO enzyme activity, we expressed the cDNA in eukaryote cells. The full-length apoxin I ORF was inserted into a pCG mammalian expression vector with an HA tag sequence at the 3′ end. When the cDNA was transfected into 293T cells, the protein was expressed in the membrane and soluble fractions of the cells, and a significant amount of the protein was secreted into the medium, as determined by anti-HA and anti-apoxin antibodies (Figure 2).

Using the recombinant apoxin I protein expressed in 293T cells, we examined the oxidase activity against L- and D-leucine. As shown in Figure 3, medium containing the secreted apoxin I protein showed the oxidase activity against L-leucine (Figure 3A), but not against D-leucine (Figure 3B), while the medium of mock-transfected 293T cells did not show L- or D-amino acid oxidase activity. Furthermore, the secreted recombinant apoxin I protein immunoprecipitated with anti-HA antibody also showed oxidase activity against L-amino acid but not against D-amino acid (data not shown). These results indicate that the recombinant apoxin I protein secreted into the medium has an LAO activity as does the protein purified from snake venom (*2*). Interestingly, the recombinant apoxin I protein kept in the transfected 293T cells showed only marginal oxidase activity against L-leucine, indicating that LAO activity of apoxin I is suppressed in the cells.

When the secreted recombinant apoxin I was added to the culture of human leukemia HL-60 cells, the cells underwent apoptosis, as shown in Figure 4A. The progression of the apoptosis was confirmed by oligonucleosomal fragmentation of chromosomal DNA (Figure 4B, lane 3). On the other hand, the recombinant apoxin I kept in the 293T cells did not induce apoptosis of HL-60 cells (lane 2), which was consistent with its marginal LAO activity (Figure 3A). Catalase (*15*) and Trolox (*16*), scavengers of hydroperoxide, inhibited apoptosis induced by recombinant secreted apoxin I, as we reported with the purified apoxin I from venom (*2*). These results indicate that the recombinant apoxin I oxidize L-amino acid to produce hydroperoxide and

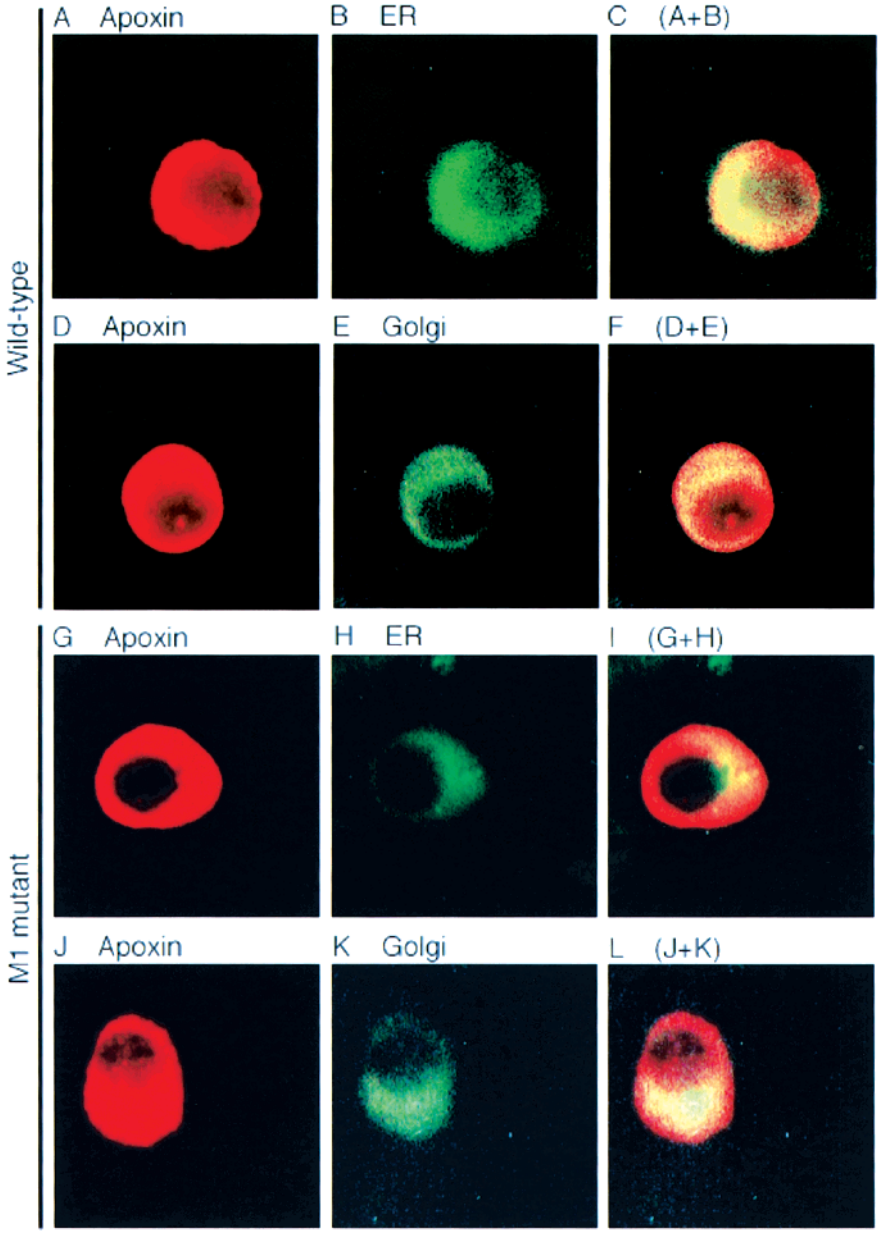

FIGURE 9: Cellular localization of apoxin I expressed in NIH3T3 cells. Transfected NIH3T3 cells were fixed and stained with reagents specific for ER (panel B and H) and trans-Golgi apparatus (panel E and K). The cells were washed and labeled with anti-apoxin I and rhodamine-conjugated anti-rabbit IgG (panel A, D, G, and J). The right panels (C, F, I, and L) are superimpositions of the left and middle panels. Yellow and orange regions in the superimposed pictures show overlapping regions of the red and green signal.

to induce apoptosis as does the natural apoxin I purified from venom.

Construction of Deletion Mutants. To examine the primary structure required for the LAO and the apoptosis-inducing activity, we constructed four deletion mutants of the apoxin I gene (Figure 5). M1 is a mutant that deleted the amino-terminal region, which is conserved in LAOs of snake venoms. M2 is the mutant that has a deletion in the highly conserved motif in A2, which is expected to bind the ADP portion of FAD. M12 is a combined version of M1 and M2, which deleted residues 2−39. The mutant M4 partially lacks the C-terminal region of A4.

Impaired Secretion and LAO Activity of Apoxin I Mutants. The wild and deletion mutants of the apoxin I gene were transfected into human 293T cells. Then, the conditioned media, membrane, and soluble fractions of the cells were prepared after 48 h. Western blot analysis with an anti-apoxin I antibody showed that all apoxin I mutants were expressed in the cells (Figure 6), a major portion in the membrane fractions (lanes 13−18) and a small portion in the soluble fractions (lanes 7−12). However, secretion of the protein into the media was strongly suppressed in cells transfected with the mutant apoxin I genes (lanes 2−6).

We next measured LAO activity of the mutant proteins in each fraction. The wild apoxin I protein secreted in the media showed LAO activity, and the wild apoxin I kept in soluble and membrane fractions of the transfected cells showed only marginal LAO activity, as described above. The mutant proteins, however, did not show LAO activity in any fractions. Since secretion of the mutant proteins into the media was greatly reduced as compared with the wild apoxin I protein, we adjusted the amount of recombinant apoxin I

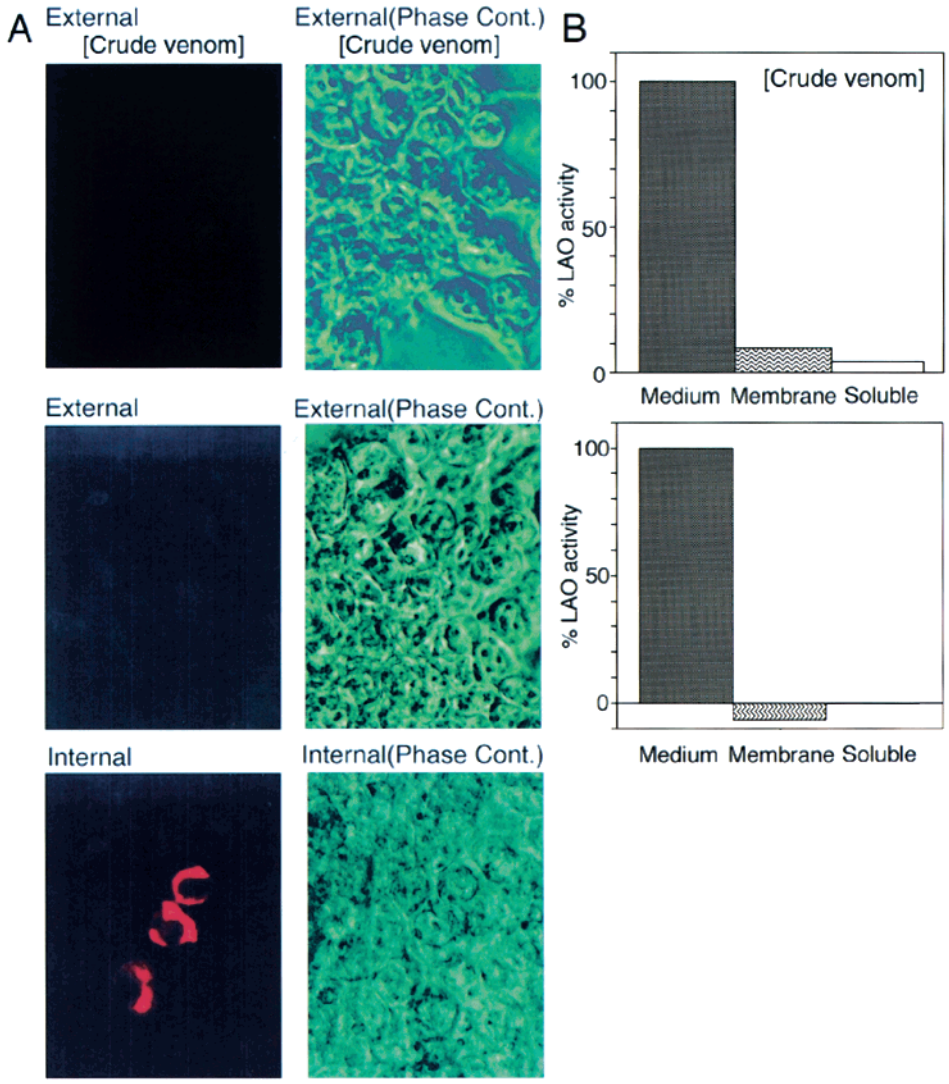

FIGURE 10: External addition of apoxin I. (A) Crude venom (Sigma V7000, from *Crotalus atrox*) was added to untreated 293T cells and incubated for 1 h in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum at 37 °C (upper panel). Active apoxin I secreted in the medium of transfected 293T cells was concentrated 10-fold and added to untreated 293T cells for 4 h in the medium (middle panel). After removal of the medium, cells were stained with anti-apoxin I antibody (left panels). As a positive control, apoxin I-transfected 293T cells producing apoxin I internally are also shown (lower panel). The same regions were photographed with phase contrast (right panels). (B) The conditioned media of cells incubated with crude venom (upper panel) and recombinant active apoxin I (lower panel) in panel A were harvested. The cells were detached from the plates with mild pipetting, then washed and lysed with sonication to prepare the membrane and soluble fractions. The LAO activity in the medium fraction is shown as 100%, and the percentage of activity of each fraction is indicated.

protein in the medium fractions and measured the LAO activity again. None of the mutants, however, showed LAO activity (lanes 19−23).

When 293T cells were transfected with wild apoxin I, a significant number of the cells died during the 48 h (Figure 7), probably due to the accumulation of the "toxic" wild apoxin I protein in the medium. However, transfection of the mutant apoxin I into 293T cells did not show any toxicity to the cells even if the culture was extended to 4 days. This finding is consistent with the defect of LAO activity in mutant apoxin I proteins. These results indicate that deletion of the amino-terminal region (A1), ADP-binding domain (A2), and carboxy-terminal region (A4) affected protein secretion and abolished LAO activity of the apoxin protein.

N-Glycosylation Is Required for Secretion and Activity of Wild Apoxin I. Apoxin I contains the Asn361-Phe-Thr sequence (Figure 1A), a potential N-glycosylation site (*17*). To examine the effect of glycosylation on secretion and LAO activity, we added tunicamycin (*18*) to the medium of wild apoxin I transfected cells (Figure 8). Tunicamycin inhibited secretion of wild apoxin I and abolished the LAO activity of the residually secreted wild apoxin I, suggesting that N-glycosylation is needed for both secretion and activity. This result is consistent with the fact that recombinant apoxin I produced in *Escherichia coli* did not show any oxidase activity (data not shown).

Immunofluorescence Study of Apoxin I. The major portion of the apoxin I protein expressed in eukaryotic cells localized in membrane fractions of the cells (Figures 2 and 6). To examine further the intracellular localization of apoxin I, immunohistochemical analysis was carried out (Figure 9). Cells were stained with $DiOC_6$(3) specific for ER or with NBD C_6-ceramide, specific for trans-Golgi apparatus and double stained with anti-apoxin I antibody. The recombinant

apoxin I protein was stained in the extranuclear region of the transfected cells (Figure 9, panels A and D). Superimposing the anti-apoxin I staining onto $DiOC_6$(*3*) staining (panel B) or NBD C_6-ceramide staining (panel E) gave yellow and orange regions (panel C and F), suggesting that apoxin I was present in ER and Golgi apparatus, respectively. The red region in the superimposed pictures may show the cytosolic localization of the apoxin I. The introduction of mutations did not change the localization of apoxin I, as represented by M1 mutant (panel G−L). These results, together with the western blot analysis, indicate that the apoxin I expressed in eukaryote cells localizes in intracellular membrane and cytosol but not in nuclei.

Externally Added Apoxin I Does Not Associate with Target Cells. A snake venom LAO was preliminary reported to accummulate into plasma membrane of cultured cells when added to the culture media (*19*). Therefore, we next asked if the active apoxin I could associate with the target cells to induce apoptosis. As shown in Figure 10A by immunostaining, the natural apoxin I (upper panel) and recombinant apoxin I (middle panel) added to culture media did not associate with the cells. The lower panel shows a positive control, i.e., the accumulation of apoxin I in the cells transfected with the apoxin I gene. To confirm this result, we prepared the medium, cellular membrane, and cytosolic fractions after treatment and measured the LAO activity in each fraction. Almost all LAO activity was recovered in the medium (Figure 10B), indicating that apoxin I added to the medium was not incorporated into the target cells.

DISCUSSION

We have cloned the cDNA of apoxin I, an apoptosis-inducing factor with LAO activity, in Western diamondback rattlesnake venom. To study the molecular function of apoxin I, we produced recombinant apoxin I. Originally we tried to produce the recombinant protein in *E. coli*. Although the transformed *E. coli* could produce the recombinant apoxin I protein in soluble fraction, the protein did not show activity to oxidize L-amino acids (data not shown). We then tried to produce the recombinant protein in the eukaryote system and found active recombinant protein in the culture medium. The recombinant protein in the cells did not show LAO activity, suggesting that apoxin I must be matured or secreted, or both, to show its activity. We do not know how the apoxin I-producer cells in the snake's venom gland control LAO activity in the cells, but it is advantageous to keep the protein inactive in the cells and to activate it, along with the protein secretion, to protect the producer cells from cytotoxic effects of apoxin I. N-Glycosylation is a possible modification involved in the maturation of the apoxin I protein because tunicamycin inhibited the secretion and emergence of LAO activity of the recombinant apoxin I protein. Further studies should be done to clarify whether N-glycosylation is directly required for subsequent secretion and apoxin I activity or required for subsequent trafficking and other posttranslational modification needed for apoxin I activity. The structural analysis on the carbohydrate chain in the native apoxin I is now in progress. The amino acid sequence shows that apoxin I has predicted FAD binding domains. Apoxin I is likely to bind FAD because LAO from *C. adamanteus*, which is highly homologous to apoxin I, can bind FAD to catalyze oxidative deamination of L-amino acids (*9*). Consistently, the deletion of an FAD binding domain (A2) abolished the LAO activity and protein secretion, suggesting that this domain is essential to show its LAO activity with FAD. Deletion of other regions (A1 and A4) also affect the secretion and LAO activity of the recombinant proteins. These regions could also have an important role in revealing LAO activity since they are also conserved in other LAOs.

The activity of apoxin I that was added externally was recovered in the medium fraction but not in soluble and membrane fractions (Figure 10). The immunofluorescence study indicated that apoxin I added externally was not internalized. In addition, flow-cytometric analysis showed that active apoxin I did not associate with the cell surface (data not shown). Thus, the major portion of active apoxin I, added externally, did not associate with cellular plasma membranes. These results suggest that active apoxin I stays in the medium and oxidizes L-amino acid to produce hydroperoxide, which damages the target cells to induce apoptosis.

Apoxin shares homology with mouse interleukin 4-induced F1G1 protein (*14*), whose function has not yet been clarified. Considering that the mammalian homologue of biologically active peptides found in venom, such as nerve growth factor (*20*, *21*), plays a physiological role, it is tempting to speculate that the mammalian homologue of apoxin I could have a physiological function by catalyzing an oxdative reaction of an endogenous substrate.

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