Regulation of a Novel Gene Encoding a Lysyl Oxidase-related Protein in Cellular Adhesion and Senescence*

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We report here a novel cDNA clone with a predicted protein sequence similar to lysyl oxidase. This full-length cDNA clone of 3432 base pairs (WS9-14) was isolated from human fibroblasts on the basis of its overexpression in senescent cells. It encodes an 87-kDa polypeptide, whose protein is a member of the scavenger receptor cysteine-rich family, because it contains four scavenger receptor cysteine-rich domains that are found in several secreted or cell surface proteins. The WS9-14 protein has a high identity with both lysyl oxidase and lysyl oxidase-like protein at a region corresponding to exons 2–6, implying the existence of a lysyl oxidase gene family. The pattern of WS9-14 gene expression by fibroblasts parallels pro-collagen I-oxidase gene family. The pattern of WS9-14 gene expression and quantitative alterations in the components of extracellular matrix proteins is altered in senescent fibroblasts (1), suggesting that the regulation of genes encoding extracellular matrix proteins is partly homologous to the gene encoding lysyl oxidase. Lysyl oxidase (LO; EC 1.4.3.13) is an extracellular, copper-dependent enzyme that initiates the cross-linking of collagens and elastin by catalyzing the oxidative deamination of peptidyl lysine to α-aminoacidipic-δ-semialdehyde. This cross-linking converts the soluble monomers of collagen and elastin into insoluble fibers in the extracellular matrix (3). Interestingly, LO also plays a role in tumor suppression in that its down-regulation is required for ras-induced cellular transformation of NIH3T3 cells (4, 5). A new gene that shows significant homology to but is distinct from the LO gene has been isolated and designated as the lysyl oxidase-like (LOL) gene (6, 7). Because the WS9-14 gene is homologous to the LO and LOL gene and is overexpressed in senescent fibroblasts, the gene product may play an important role in the age-associated changes in extracellular matrix proteins. Here we describe the isolation of the full-length WS9-14 cDNA, its structure, and expression.

EXPERIMENTAL PROCEDURES

Isolation of 5'-Upstream Region of the cDNA—Polymerase chain reaction (PCR) was used to amplify the 5'-upstream region of the WS9-14 DNA fragment. First, the 5' DNA fragments were amplified from the Basinger cDNA library (a gift from Dr. H. Okayama), derived from normal human fibroblasts, by using the sense primer SG4 (5'-TCTAG-GCCGTGAGGGAA-3', specific for bases at position 30–47 upstream of the cloning site of the vector) and antisense primer HS1 (5'-CAGTAGCCGTCTCTCCTC-3', located at bases 60–77 from the 5'-end of the initial WS9-14 clone). The PCR products were ligated into pITBlue vector (Novagen) and cloned in Escherichia coli DH5α. The nucleotide sequences were determined. Next, to isolate the 5'-end of the cDNA, 5' rapid amplification of cDNA end (5'-RACE) (8) was carried out using a commercial kit (Life Technologies, Inc.). Antisense primer HS4 (5'-TGATGTCGTCAAGGGTGCGC-3', specific for the bases at 466–484 site downstream of the beginning of the newly isolated WS9-14 DNA fragment) was used to synthesize first strand cDNA from 1 μg of total RNA derived from human skin fibroblasts GM37. For PCR amplification, the sense Universal Amplification Primer (Life Technologies, Inc.) and antisense primer HS5 (5'-AGCACAGAGGCCTCTCCAT-3', specific for bases 70–88 downstream of the 5'-end of the newly isolated WS9-14 fragment) were used (Fig. 1A). The PCR products were cloned, and nucleotide sequences were determined. A few PCR errors were found and eliminated from the sequencing data by performing triplicated PCR amplification and comparing the nucleotide sequences derived from each PCR.

DNA Sequencing—A series of nested deletion mutants of WS9-14 cDNA was made by controlled exonuclease III digestion using a Nested Deletion kit (Pharmacia Biotech Inc.). Nucleotide sequences of both strands were determined by the dye-exchange chain termination method using the Sequanase T7 DNA polymerase (U. S. Biochemical Corp.) according to the manufacturer's protocol.

Cell Lines—All cell lines used in this study were derived from human subjects. IMR90 (fetal lung fibroblast strain) was obtained from the NIA Aging Cell Repository; N90 was established by immortalizing IMR90 with SV40 large T antigen (9); GM37 (skin fibroblast strain), GM37 (SV40-transformed GM37), and GM1815 (Epstein-Barr virus-transformed GM1815) were a gift from Dr. H. Okayama, derived from normal human fibroblasts, by using the sense primer SG4 (5'-TCTAG-GCCGTGAGGGAA-3', specific for bases at position 30–47 upstream of the cloning site of the vector) and antisense primer HS1 (5'-CAGTAGCCGTCTCTCCTC-3', located at bases 60–77 from the 5'-end of the initial WS9-14 clone). The PCR products were ligated into pITBlue vector (Novagen) and cloned in Escherichia coli DH5α. The nucleotide sequences were determined. Next, to isolate the 5'-end of the cDNA, 5' rapid amplification of cDNA end (5'-RACE) (8) was carried out using a commercial kit (Life Technologies, Inc.). Antisense primer HS4 (5'-TGATGTCGTCAAGGGTGCGC-3', specific for the bases at 466–484 site downstream of the beginning of the newly isolated WS9-14 DNA fragment) was used to synthesize first strand cDNA from 1 μg of total RNA derived from human skin fibroblasts GM37. For PCR amplification, the sense Universal Amplification Primer (Life Technologies, Inc.) and antisense primer HS5 (5'-AGCACAGAGGCCTCTCCAT-3', specific for bases 70–88 downstream of the 5'-end of the newly isolated WS9-14 fragment) were used (Fig. 1A). The PCR products were cloned, and nucleotide sequences were determined. A few PCR errors were found and eliminated from the sequencing data by performing triplicated PCR amplification and comparing the nucleotide sequences derived from each PCR.

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Novel Lysyl Oxidase-related cDNA

**RESULTS AND DISCUSSION**

Although the WS9-14 transcript was previously reported as a 4.2-kb RNA (2), Northern blot analyses of additional RNA samples detected a 3.65-kb single band (Fig. 2). The complete sequencing of the previously isolated WS9-14 clone revealed that it was a 2486-bp fragment. An additional 768-bp fragment of 5′-upstream region was isolated by PCR from another fibroblast cDNA library (Fig. 1A). This fragment was then used in 5′-RACE procedure to obtain the 5′-end of the cDNA. This resulted in the cloning of a single PCR product that contained an additional 178-bp fragment of WS9-14 cDNA (Fig. 1B). The first 22 amino acids of the signal peptide sequence are designated N, residues that are common in any two of the three proteins are depicted as bold letters, and the cleavage site is indicated by an arrow. Cysteine residues are circled. The four SRCR domains are linked carbohydrate could be attached (Asn-Xaa-Ser/Thr). The context of the first ATG codon, GGGATGG, matches the Kozak consensus sequence for an optimal translation initiation site at +1 to a stop codon at position 248 from the 5′-end of the cDNA. This 4.2-kb mRNA overexpressed in human fibroblasts at late passages. Total RNA from IMR90 cells at population doubling levels (PDL) 25 and 65 was compared by Northern blot analysis.

**FIG. 2.** The 3.65-kb WS9-14 mRNA overexpressed in human fibroblasts at late passages. Total RNA from IMR90 cells at population doubling levels (PDL) 25 and 65 were compared by Northern blot analysis.
have been implicated in the post-transcriptional regulation of mRNA stability (13). The 22 amino acids, starting from the first AUG initiation codon, possess features characteristic of signal peptide sequences (14), suggesting that WS9-14 is an extracellular protein. Cleavage of the signal peptide would yield a protein of 749 amino acids with three potential N-linked glycosylation sites and 34 cysteine residues (Fig. 1B).

A search of the GenBank® and EMBL data base revealed that the amino acid sequence from 546 to 751 of WS9-14 showed a 48% identity to the C termini of both LO and LOL (Fig. 1C; 6, 7, 15, 16). This region corresponds to exons 2–6 in the LO gene. Both LO and LOL share 76% homology at this region. Thus, there may be a lysyl oxidase family consisting of genes that share homology in this region. There are 10 cysteine residues in this region, and all of them are conserved in the three proteins. Although this homologous region of over 200 amino acids is at the C terminus of both LO and LOL, there is an additional unique sequence of 23 amino acids at the C terminus of WS9-14. Another homologous region involves 8 among 11 amino acid residues in the putative copper binding site in human LO (17) that are conserved in WS9-14 including the four histidine residues that are presumably supplying the nitrogen ligands for copper coordination. Furthermore, another putative copper binding site glycy1-histidyl-lysyl (GHK), which is a collagen-related copper affinity site, is also conserved in WS9-14 (Fig. 1C). These data suggest that WS9-14 may also be a copper-binding protein. Recent, a quinone cofactor was identified in the active site of LO. It is derived from a cross-linking between a Tyr derivative and a Lys residue in LO and is thus designated lysine tyrosylquinone (18). In WS9-14, these Lys and Tyr residues are conserved, suggesting that WS9-14 may also form such a quinone cofactor by cross-linking at the same site (Fig. 1C). These structural similarities of WS9-14 and LO raise the possibility that WS9-14 may have a LO-like activity such as cross-linking of extracellular matrix.

Amino acid sequence alignment revealed that WS9-14 contains four repeats of the scavenger receptor cysteine-rich (SRCR) domains (19) at amino acids 58–158, 190–301, 326–424, and 435–543 (Fig. 1B). SRCR domains contain 6–8 conserved cysteine residues over a 100-amino acid stretch and have been found in diverse secreted and cell membrane-associated proteins such as macrophage scavenger receptor type I (20) and lymphocyte glycoproteins CD5 (21) and CD6 (22). Thus, the presence of the SRCR domains, as well as a signal sequence in WS9-14, suggests that it is an extracellular protein. Although the function of the SRCR domains is not clear, they are likely to be involved with binding to other cell surface or extracellular molecules. The SRCR domains form most of the extracellular sequence of CD5 (21), which binds to its ligand, CD72 (23). Furthermore, the membrane-proximal SRCR domains in CD6 are necessary for binding of CD6 to its ligand (24).

Our previous study suggested that WS9-14 and pro-collagen I-α1 have a parallel expression pattern in fibroblasts following subculture and proliferation from sparse to confluent cultures (2). We felt it was relevant, therefore, to compare levels of expression of these two genes by fibroblasts in culture in the presence of TGF-β1 or indomethacin, which increase procollagen-I transcripts (25, 26), and in the presence of phorbol ester or retinoic acid, which inhibit procollagen-I gene expression (27, 28). As shown in Fig. 4A, WS9-14 mRNA levels increased by TGF-β1 and indomethacin and decreased by treatment with phorbol ester and retinoic acid. This similarity of gene expression suggests the existence of multiple common regulatory elements in the WS9-14 and procollagen promoter regions. TGF-β1 is known to increase the mRNA levels of many extracellular matrix components such as collagens and fibronectin (25). Our results, therefore, suggested that WS9-14 is an extracellular matrix component.

Comparison of WS9-14 mRNA levels in various human tumor cell lines has shown that the RNA levels differ significantly in adherent cell lines and suspension cell lines. The WS9-14 mRNA was abundant in the seven adherent cell lines N90, U373MG, HT1080, HeLa (Fig. 4B), HuTu60, Hs294T, and GM637 (data not shown). In contrast, the WS9-14 mRNA was absent or barely detectable in the six suspension cell lines HeLa-S3, K562, Jurkat, KATO III, and NCI-H69 (Fig. 4B) and GM1815 (data not shown). This correlation of WS9-14 expression and cell adhesion phenotype suggests that the WS9-14 protein may be involved in the cell adhesion and that loss of WS9-14 expression in tumor cells may be associated with loss of adhesion and therefore play a role in metastasis. Because WS9-14 is likely to be an extracellular protein with structural similarity to lysyl oxidase, its function may involve post-translational modification of extracellular matrix components or other cell membrane-associated proteins. Such modifications could modulate matrix-cell communication, which is important to the cell adhesion phenotype.
The cross-linking theory of aging implies that a progressive accumulation of intermolecular cross-linking of macromolecules causes deleterious effects in aged animals. Extensive cross-linking in collagen could decrease its solubility, elasticity, and permeability, which would increase viscosity in the extracellular compartment, thereby impairing the flow of nutrients and waste products into and out of cells (29). Indeed, cross-linking of human and bovine skin collagen increases with age (30). Markedly increased expression of LO is found in pulmonary, arterial, dermal, and liver fibrosis (31), pathologies that are often associated with aging. Therefore, the overexpression of WS9-14 mRNA in senescent fibroblasts (Fig. 2) may also be related to these age-associated pathophysiological characteristics.

In conclusion, the novel cDNA WS9-14 isolated from a senescent fibroblast cDNA library encodes a protein with structure similar to LO and LOL. Although the function of this protein is currently unknown, our data suggest that it may be related to age-associated pathologies involving extracellular matrix cross-linking and cellular adhesion.

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