Rat FAD-dependent sulfhydryl oxidase was purified; partial sequencing indicated that it was homologous to human quiescin Q6. A cDNA (GenBank™ accession no. AF285078) was cloned from rat seminal vesicles, and active recombinant sulfhydryl oxidase was expressed in Chinese hamster ovary epithelial cells. This 2472-nucleotide cDNA has an open reading frame of 1710 base pairs, encoding a protein of 570 amino acids including a 32-amino acid leader sequence and two potential sites for N-glycosylation. One of them is used and the 64,000 M_r purified protein was transformed to 61,000 by the action of endoglycosidase F. Northern blotting and reverse transcription-polymerase chain reaction analyses showed that there were small amounts of sulfhydryl oxidase in the rat testis, prostate, lung, heart, kidney, spleen, and liver, and that the gene was highly expressed in seminal vesicles and epididymides. Rat sulfhydryl oxidase cDNA corresponds to the human cell growth inhibiting factor cDNA, which could be a differently spliced form of quiescin Q6. Comparing sulfhydryl oxidase sequences with those of human quiescin Q6 and mammalian and Caenorhabditis elegans quiescin Q6-related genes established the existence of a new family of FAD-dependent sulfhydryl oxidase/quiescin Q6-related genes containing protein-disulfide isomerase-type thioredoxin and yeast ERV1 domains.

Sulfhydryl oxidases are enzymes that catalyze the reaction 2R-SH + O_2 → R-S-S-R + H_2O_2. There are at least three families of these enzymes, each depending on different cofactors for their catalytic activity. They are iron-dependent sulfhydryl oxidases (1–4), copper-containing enzymes (5–8), and FAD-dependent enzymes. The last of these have been found in seminal vesicle fluids from rats and hamsters (9, 10). Rat sulfhydryl oxidase was purified from seminal vesicles. It is a monomeric enzyme with an apparent M_r of 66,000, a pH of 7.45, and 1 mol of noncovalently bound FAD/mol of enzyme (14). The rat enzyme accepts a variety of small sulfhydryl substrates including glutathione, cysteine, dithiothreitol (DTT), and 2-mercaptoethanol and can also markedly enhance the rate of renaturation of fully reduced ribonuclease (14). Several possible functions have been proposed for sulfhydryl oxidase in the rat male genital tract. These include the generation of disulfide bonds in the proteins of seminal plasma or spermatozoa, the preservation of spermatozoan membrane integrity, antimicrobial activity (through the release of H_2O_2), and the protection of spermatozoa against the harmful effects of thiol after ejaculation (14). It has been shown recently that flavin-dependent sulfhydryl oxidase from chicken egg white contains one redox-active cystine bridge, and accepts a total of 4 electrons per active site (15). This oxidase has a high catalytic activity toward reduced peptides and proteins including insulin A and B chains, lysozyme, ovalbumin, riboflavin-binding protein, and RNAse (16). Flavin-dependent chicken egg white sulfhydryl oxidase and protein disulfide isomerase can also cooperate in vitro in the generation and rearrangement of native disulfide pairings (16).

Neither protein nor nucleotide sequences were available for FAD-dependent sulfhydryl oxidases until the partial sequencing of chicken egg white sulfhydryl oxidase (17) (published after the original submission of this work) and rat sulfhydryl oxidase (this work). These sequences were similar to those of four mammalian cDNAs. The first of these, encoding human bone-derived growth factor-1 (BPGF-1; GenBank™ accession no. L42379) was cloned from an osteosarcoma cell line. The second, GEC-3 (GenBank™ accession no. U82982), was the product of a gene whose expression is hormone-dependent in the uterine tissue of the guinea pig (Cavia porcellus). The third, encoding the cell growth inhibiting factor (CGIF; GenBank™ accession no. E12644) was cloned from human lung fibroblast. The last is the product of quiescin Q6 (GenBank™ accession no. E12644). The abbreviations used are: DTT, dithiothreitol; BPGF-1, human bone-derived growth factor-1; CGIF, cell growth inhibiting factor; DTNB, 5,5’-dithiobis-2-nitrobenzoic acid; E-64, L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane; ERV-1, essential for respiration and viability-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEC-3, guinea pig endometrial cell-3; hQ6, human quiescin Q6; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; PDI, protein-disulfide isomerase; PAGE, polyacrylamide gel electrophoresis; SOX, sulfhydryl oxidase; Z-Phe-Arg-NH-Mec, carbobenzoxy-l-phenylalanyl-l-arginine-4-methylcoumarinyl-7-amide; Mec, 4-methyl-7-coumarylamide; DEMEM, Dulbecco’s modified Eagle’s medium; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s).
Papain inhibitory activity was in the flow-through of the column. The inhibitory activity eluted with a gradient of urea (0.0–6.0 M) in 0.01 M potassium phosphate buffer, pH 6.8, and loaded on to a phenyl-Sepharose column (Amersham Pharmacia Biotech) to remove the glycyl-endopeptidase (EC 3.4.22.25) which usually contaminates the commercial enzyme. The column was then washed with 30% ethylene glycol and the inhibitory fractions were quantified by densitometry and corrected using GAPDH as internal standard.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis—The first cDNA strand was synthesized from 5 μg of total RNA prepared from various rat tissues using Moloney murine leukemia virus reverse transcriptase (Promega) and oligo(dT)17 primer, following the manufacturer's instructions. At the end of reverse transcription, the reaction mixture (20 μl) was diluted to 1000 μl with 10 mM Tris, pH 7.5, 1 mM EDTA and 1 μl of this solution was used to amplify a 400-bp SOx fragment or a 800-bp GAPDH fragment (internal standard). The sequences of the 5’ and 3’ primers for SOx were 5’-ACCTGAGGAGTT-GACAGTCAG-3’ and 5’-AGCAGACCTAGCGGGA-3’. For GAPDH they were 5’-AGCTCAACGGCAGCTAAGCTGATT-3’ and 5’-GGAGGGCCTCACCAGTTCGATGGC-3’. Amplification was performed in a Pneogen thermocycler (Techne) using Platinum® Pfx DNA polymerase (Life Technologies, Inc.). After one cycle at 94 °C for 2 min, 62 °C for 2 min, and 68 °C for 40 min for second strand synthesis, the thermocycling parameters were 30 cycles of amplification at 96 °C for 10 s, 59 °C for 30 s, and 68 °C for 30 s. Ten microliters (1/5 of PCR products) were analyzed on 1% agarose gel. Expression of SOx mRNA was quantified by densitometry and corrected using GAPDH as internal standard.
was used to measure recombinant sulfhydryl oxidase activity, as de-
The H₂O₂ produced was measured using DTNB. The activity of papain incubated with sulfhydryl oxidase. Papain (0.01 mM) was incubated with sulfhydryl oxidase in 0.1 M phosphate buffer, pH 6.8, 1 mM EDTA, 0.1% Brij 35, and 2 mM DTT at 37 °C. Activity of the papain (0.25 μM final) was analyzed at different times with 5 μM Z-Phe-Arg-NH-Mec. Fluorescence is expressed as arbitrary units (AU). The H₂O₂ concentration was deduced from the free sulfhydryl concentration determined using DTNB. The H₂O₂ produced was calculated on the basis of 1/2 H₂O₂ per R-SH consumed by sulfhydryl oxidase.

Compared with the deduced protein sequence, the higher mass of the native form is probably due to the carbohydrate content of the mature protein, which is glycosylated, as seen in Fig. 1. The rat sulfhydryl oxidase contains a signal peptide, which leads to a mature protein that is glycosylated, as seen in Fig. 1. The native enzyme, which is 64,624 by mass spectrometry (Fig. 1), seems to be used to obtain the complete cDNA of sulfhydryl oxidase from seminal vesicles using a primer based on the homology between the amino-terminal sequence of the rat enzyme and the deduced open reading frame of human quiescin Q6, guinea pig GEC3, and human BPGF-1 cDNA (as described under "Experimental Procedures").

The rat 2472-bp sulfhydryl oxidase cDNA contains a polyadenylation signal after the stop codon (Fig. 3) showing that it codes for a full-length sulfhydryl oxidase. This cDNA has an open reading frame of 1710 bp coding for 570 amino acids. The deduced protein contains a 32-amino acid putative signal peptide, which leads to a mature protein with a calculated Mr of 60,035, a value distinct from that of the native enzyme, which is 64,624 by mass spectrometry (Fig. 1). Compared with the deduced protein sequence, the higher mass of the native form is probably due to the carbohydrate content of the mature protein, which is glycosylated, as seen in Fig. 1. The rat sulfhydryl oxidase contains a signal peptide, which leads to a mature protein that is glycosylated, as seen in Fig. 1. The native enzyme, which is 64,624 by mass spectrometry (Fig. 1), seems to be used to obtain the complete cDNA of sulfhydryl oxidase from seminal vesicles using a primer based on the homology between the amino-terminal sequence of the rat enzyme and the deduced open reading frame of human quiescin Q6, guinea pig GEC3, and human BPGF-1 cDNA (as described under "Experimental Procedures").

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sequences associated with the sulfhydryl oxidase activity of rat protein favor the definition of a new sulfhydryl oxidase/quiescin Q6 gene family characterized by the original juxtaposition of thioredoxin and ERV1 domains.

Expression and Enzymatic Activity of Recombinant Rat Sulfhydryl Oxidase in CHO Cells—The rat sulfhydryl oxidase cDNA was stably expressed in CHO epithelial cells. This cell line, which has no endogenous sulfhydryl oxidase secretion (Fig. 6A), was transfected with the pCR3.1-rSOx plasmid. After 2 weeks in G418 selective medium, resistant colonies were selected and serially passaged. Four stably transfected colonies were assayed for recombinant sulfhydryl oxidase expression. Recombinant protein was immunoprecipitated from transfected cell supernatants or whole cell extracts with immune rat sulfhydryl oxidase serum and subjected to Western blot analysis. Immunoprecipitation revealed a $M_r$ 64,000 band in the supernatant of rSOx-transfected CHO cells, which was lacking in the supernatant of nontransfected cells and pcDNA3.1-LacZ-transfected cells (Fig. 6A). The electrophoretic mobility of the recombinant protein was identical to that of native enzyme from seminal vesicles. Finally, any expression of rat recombinant rat sulfhydryl oxidase protein was observed in immunoprecipitation from transfected whole cell extracts (data not shown); this confirms that sulfhydryl oxidase protein is mainly secreted.

Oxidative activity of recombinant protein in the supernatant of stably transfected CHO cells was measured using DTT as a substrate (Fig. 6C). Sulfhydryl oxidase activities correlated with the expression level of recombinant protein, as shown in the Western blot experiment (Fig. 6A). Activity due to recombinant protein in clone 4 supernatant corresponded to the activity of 75 ng of pure native enzyme measured in the same experimental conditions. These data show that recombinant sulfhydryl oxidase secreted by stably transfected CHO cells
Cloning of Rat Seminal Vesicle FAD-dependent SOx

FAD-dependent sulfhydryl oxides have previously been identified and characterized by their enzymatic activities and their biochemical properties in several tissues and species including rat seminal vesicles (9, 11, 14), hamster seminal vesicles (10), chicken egg white (13), and human platelets (31). Although sulfhydryl oxidases were thought to be members of the pyridine nucleotide disulfide oxidoreductases (32), no data on the nucleotide or amino acid sequences of these molecules were available in the literature. Purification and partial sequencing of rat FAD-dependent seminal vesicle fluid sulfhydryl oxidase and molecular cloning of the corresponding cDNA, indicated a great similarity at the nucleotide and the amino acid levels with human quiescin Q6 and guinea pig GEC-3 cDNA. All three deduced protein sequences possessed a putative peptide signal, two conserved potential N-glycosylation sites, and putative thioredoxin and ERV1 domains. Such structural homologies among these different molecules argue strongly in favor of a new gene family and confirm the preliminary work of Hoober et al. (17) on egg white sulfhydryl oxidase and quiescin Q6. This family also includes CeQ6-related sequences of C. elegans and Drosophila (18). Because the quiescin behavior (i.e. induction of gene expression that takes place when cells enter the quiescent phase) seems thus far to be restricted to human quiescin Q6 cDNA (19, 33), this family should be named the sulfhydryl oxidase/quiescin Q6 family until the enzymatic activity of its members, and their behavior during the proliferative cycle, has been fully determined.

Two quiescin Q6 transcripts of 2500 and 3200 nucleotides have simultaneously been found in the W138 human lung fibroblast cell line (where only the longer form has been cloned) (18) and are also present in several of the cell lines or tissues analyzed (33). Furthermore, two human quiescin Q6-related cDNAs, bone-derived growth factor-1 and cell growth inhibiting factor, have also been independently cloned; they show 98% identity with human quiescin Q6, although a few differences cause modifications in their putative open reading frames. These differences could be due to PCR or sequencing errors as well as to real gene modifications. The 3228-nucleotide BPGF-1 cDNA was cloned from an osteosarcoma cell line and seems to correspond to quiescin Q6 cDNA (3298 nucleotides). The 2500-nucleotide CGIF cDNA was isolated from another lung fibroblast cell line (MRC-5), and but it lacks a 728-nucleotide segment (1879–2606 of quiescin Q6) in its 3'-noncoding sequence. CGIF cDNA could therefore represent an alternative transcript of quiescin Q6. Rat sulfhydryl oxidase cDNA (2472 nucleotides), which lacks the same 3'-noncoding 728-nucleotide segment, appears therefore to be the homologue of human cell growth inhibiting factor cDNA.

An analysis of the expression pattern of rat sulfhydryl oxidase...
by Northern blotting indicated that the gene is actively expressed in the epididymis and seminal vesicles. This was confirmed by RT-PCR and agree with the distribution of sulfhydryl oxidase activity in the rat and guinea pig tissues, showing low activity in testis, more activity in the epididymis, and the greatest activity in seminal vesicles, but no activity in muscle, brain, kidney, liver, or lung tissue (9, 10). On the other hand, RT-PCR and Northern blot revealed low expression of the rat sulfhydryl oxidase gene in all the tissues analyzed. This corresponds to the wide variety of human tissues expressing human quiescin Q6, including the heart, placenta, lung, liver, skeletal muscle, and pancreas (33). Therefore, apart from the high concentration of sulfhydryl oxidase in the male reproductive tract of rodents, sulfhydryl oxidase and quiescin Q6 appear to be ubiquitous.

Although this molecule is clearly a secretory protein, sulfhydryl oxidase immunoreactivity has also been found in the matrix of the mitochondria of certain human, rat, and hamster testicular cells at specific stages of functional activation (34–36). In rat and hamster, this immunoreactivity appears in pachytenic spermatocytes at stage I (34). In photoperiodically-induced testicular involution in the Djungarian hamster, immunoreactivity reappeared during recrudescence, when the first spermatogenic wave had reached the pachytenic stage (37). In mature human testis, moderate sulfhydryl oxidase immunoreactivity has been found in Leydig cells and spermatogonia in and pachytenic spermatocytes (36). Sulfhydryl oxidase also seems to be associated with hypospermatogenesis and impaired fertility (38). Sulfhydryl oxidase appears therefore to be implicated in the functional or the activation state of mitochondria, at least in the cells of the mammalian male reproductive tract.

The ERV1 gene is essential for mitochondrial biogenesis and the survival of Saccharomyces cerevisiae cells. ERV1 is found in the cytosol and mitochondria of the yeast cell where it plays an essential role in normal mitochondrial morphology and the stability of these organelles. ERV1p has been recently described as a protein FAD-dependent sulfhydryl oxidase; its homologue, human augmenter of liver regeneration (ALRfp) is a mammalian hepatic growth factor that can functionally substitute for ERV1 in yeast (41) and is also probably a sulfhydryl oxidase. Therefore sulfhydryl oxidases are essential molecules in lower and higher eukaryotes implicated in mitochondrial function as well as in the cellular growth regulation, and could be important in pathological states such as cancer development.

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REFERENCES

1. Janolino, V. G., and Swaisgood, H. E. (1975) J. Biol. Chem. 250, 2532–2538
2. Isaac, C. E., Pascal, T., Wright, C. E., and Guall, G. E. (1984) Pediatric Res. 18, 532–535
3. Schulz, C., H., Swaisgood, H. E., and Horton, H. R. (1982) Biochem. Biophys. Res. Commun. 107, 196–203
4. Clare, D. A., Pinnix, I. B., Leege, J. G., and Horton, H. R. (1988) Arch. Biochem. Biophys. 265, 351–361
5. Lash, L. H., Jones, D. P., and Orrenius, S. (1984) Biochim. Biophys. Acta 779, 191–200
6. Lash, L. H., and Jones, D. P. (1986) Arch. Biochem. Biophys. 247, 120–130
7. Goldsmith, L. A. (1987) Methods Enzymol. 143, 510–515
8. Takahori, K., Thorpe, J. M., and Goldsmith, L. A. (1980) Biochim. Biophys. Acta 615, 309–323
9. Chang, T. S., and Morton, B. (1975) Biochem. Biophys. Res. Commun. 66, 309–315
10. Chang, T. S., and Zirkin, B. R. (1978) Biol. Reprod. 18, 745–748
11. Ostrowski, M. C., Kistler, W. S., and Williams-Ashman, H. G. (1979) Biochem. Biophys. Res. Commun. 87, 171–176
12. de la Motte, R. S., and Wagner, F. W. (1987) Biochemistry 26, 7363–7371
13. Hoober, K. L., Jonesa, B., White, H., III, and Thorpe, C. (1996) J. Biol. Chem. 271, 30510–30516
14. Ostrowski, M. C., and Kistler, W. S. (1980) Biochemistry 19, 2639–2645
15. Hoober, K. L., and Thorpe, C. (1999) Biochemistry 38, 3211–3217
16. Hoober, K. L., Sheasley, S. L., Gilbert, H. F., and Thorpe, C. (1999) J. Biol. Chem. 274, 22147–22150
17. Hoober, K. L., Glynn, N. M., Burns, S., Coppel, D., and Thorpe, C. (1999) J. Biol. Chem. 274, 31759–31762
18. Coppel, D. L., Cina-Poppe, D., and Gillemert, S. (1992) Genomics 14, 460–468
19. Coppel, D. L., Kopman, C., Scandalis, S., and Gillemert, S. (1993) Cell Growth Differ. 4, 483–493
20. Buttle, D. J., Kembhavi, A. A., Sharp, S. L., Shute, R. E., Rich, D. H., and Barrett, A. J. (1989) Biochem. J. 263, 451–459
21. Esnard, A., Esnard, F., and Gauthier, F. (1988) FEBS Lett. 236, 475–478
22. Esnard, A., Esnard, F., and Gauthier, F. (1988) Biol. Chem. Hoppe Seyler 369, 219–222
23. Hawke, D., and Yuan, P. (1987) Applied Biosystems Bulletin 28, Applied Biosystems, Foster City, CA
24. Esnard, A., Esnard, F., Faucher, D., Capony, J. P., Derancourt, J., Brillard, M., and Gauthier, F. (1990) J. Biol. Chem. Hoppe Seyler 371, 161–166
25. Barrett, A. J., Kembhavi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tama, M., and Hanada, K. (1982) Biochem. J. 201, 389–396
26. Ellison, G. L. (1959) Arch. Biochem. Biophys. 73, 80–72
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Frohman, M. A. (1994) PCR Methods Appl. 4, S40–S48
29. Laemmli, U. K. (1970) Nature 227, 680–685
30. Turk, V., and Bode, W. (1991) FEBS Lett. 285, 213–219

2 A. Esnard-Feve and F. Esnard, unpublished observations.
31. Kaul, D., Dhawan, V., and Kaur, M. (1996) *Mol. Cell. Biochem.* **159**, 81–84
32. Williams, C. H. J. (1990) *Chem. Biochem. Flavoenzymes III*, 121–211
33. Coppock, D., Kopman, C., Gudas, J., and Cina-Poppe, D. A. (2000) *Biochem. Biophys. Res. Commun.* **269**, 604–610
34. Kumari, M., Aumuller, G., Bergmann, M., Meinhardt, A., and Seitz, J. (1990) *Histochemistry* **94**, 365–371
35. Bergmann, M., Oehmen, F., Kumari, M., Aumuller, G., and Seitz, J. (1991) *J. Reprod. Fertil.* **91**, 259–265
36. Aumuller, G., Bergmann, M., and Seitz, J. (1991) *Cell Tissue Res.* **266**, 23–28
37. Bergmann, M., Kumari, M., Aumuller, G., Hoffmann, K., and Seitz, J. (1990) *Int. J. Androl.* **13**, 488–499
38. Bergmann, M., Aumuller, G., Seitz, J., and Nieschlag, E. (1992) *Cell Tissue Res.* **267**, 209–214
39. Lee, J., Hofhaus, G., and Lisowsky, T. (2000) *FEBS Lett.* **477**, 62–66
40. Lyles, M. M., and Gilbert, H. F. (1991) *Biochemistry* **30**, 619–625
41. Hofhaus, G., Stein, G., Polimeno, L., Francavilla, A., and Lisowsky, T. (1999) *Eur. J. Cell Biol.* **78**, 349–356